AD_____

Award Number: W81XWH-10-1-0244

TITLE: Loss of PTEN as a Predictive Biomarker of Response to Lithium Chloride, A Potential Targeted Treatment for Breast Cancer

PRINCIPAL INVESTIGATOR: Michaela Higgins

CONTRACTING ORGANIZATION: Massachusetts General Hospital
Boston, MA 02114

REPORT DATE: November 2013

TYPE OF REPORT: Annual Summary

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE *(DD-MM-YYYY)*	2. REPORT TYPE	3. DATES COVERED *(From - To)*
November 2013	Annual Summary	1 June 2010 - 31 August 2013

4. TITLE AND SUBTITLE
Loss of PTEN as a Predictive Biomarker of Response to Lithium Chloride, A Potential Targeted Treatment for Breast Cancer

5a. CONTRACT NUMBER

5b. GRANT NUMBER
W81XWH-10-1-0244

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)
Michaela Higgins

E-Mail: mjhiggins@partners.org

5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
Massachusetts General Hospital
Boston, MA 02114

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT

We overexpressed the epidermal growth factor receptor (EGFR) into the non-tumorigenic human breast epithelial cell line MCF-10A, and compared these cells to isogenic cell lines previously created via somatic cell gene targeting to model Pten loss, PIK3CA mutations, and the invariant AKT1 mutation, E17K. EGFR overexpressing clones were capable of cellular proliferation in the absence of EGF and were sensitive to lithium similar to the results previously seen with cells harboring PIK3CA mutations. In contrast, AKT1 E17K cells and PTEN -/- cells displayed resistance or partial sensitivity to lithium, respectively. Western blot analysis demonstrated that lithium sensitivity correlated with significant decreases in both PI3K and MAPK signaling that were observed only in EGFR overexpressing and mutant PIK3CA cell lines. These studies demonstrate that EGFR overexpression and PIK3CA mutations are predictors of response to lithium, whereas Pten loss and AKT1 E17K mutations do not predict for lithium sensitivity. Our findings may have important implications for the use of these genetic lesions in breast cancer patients as predictive markers of response to emerging PI3K pathway inhibitors.

15. SUBJECT TERMS
Isogenic cell lines, PTEN , PIK3CA

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			USAMRMC
U	U	U	UU	39	19b. TELEPHONE NUMBER *(include area code)*

Table of Contents

Page

Introduction……..4

Body……..4

Key Research Accomplishments……..13

Reportable Outcomes……13

Conclusion…… 13

References……16

Appendices…… 17

Introduction

The phosphatidylinositol 3-kinase (PI3K) pathway mediates key cellular functions, including growth, proliferation, survival and angiogenesis. The gene encoding the catalytic domain of PI3K, *PIK3CA*, has been found to be mutated in breast cancers at high frequency. The tumor suppressor PTEN reverses the effects of PI3K by dephosphorylating the same site on membrane phosphatidylinositols that is phosphorylated by PI3K. Genomic analysis of the PTEN gene has identified it as one of the most commonly mutated or deleted tumor suppressors in human malignancies. In breast cancer, genetic alterations of both PTEN alleles are found with a frequency of about 5%, however monoalleleic loss of PTEN is observed in as many as 50% of cases, and this can lead to aberrant PTEN signaling, resulting in early metastasis and poor prognosis.

Physiologic models of PTEN loss are needed to test potential anti-cancer therapies in preclinical animal models of breast cancer. Our lab's approach is to exploit somatic cell gene targeting to create paired isogenic cell lines with critical genetic alterations as their only differentiating factor. The changes in downstream signaling pathways can then be reliably detected, and response to new therapeutic agents can be identified. Thus, the ability to create isogenic paired human cell lines enables the evaluation of genetic alterations for predictive biomarkers of response to novel therapies.

Using gene targeting, our laboratory has introduced two common "hotspot" *PIK3CA* mutations into the MCF-10A non-tumorigenic human breast epithelial cell line. (1) Surprisingly, this study led to the discovery that GSK3beta inhibitors including lithium, an FDA approved therapy for bipolar disorders, have selective anti-neoplastic properties against human breast cancer cell lines containing oncogenic *PIK3CA* mutations. These results are now being rapidly translated into a clinical trial to determine if women with breast cancers harboring mutant *PIK3CA* will respond to lithium therapy. Because of the known opposing interactions between PI3K and PTEN, we hope to build upon this work and study the sensitivity to lithium in breast cancer cells with PTEN loss using preclinical models. If successful, we will rapidly translate these findings to an early phase clinical trial studying the safety and efficacy of oral lithium treatment in patients with breast carcinoma and examining their breast cancers for PTEN loss. We hope to provide the rationale for the use of lithium as a targeted anti-cancer treatment for breast cancer patients whose tumors harbor mutations/loss of PTEN.

Body

Statement of work

Task 1. Creation of isogenic breast cell lines with heterozygous and homozygous PTEN loss. (months 1-18)
1a. Create targeting vectors (months 1-3)
1b. Infect and screen breast epithelial and cancer cell lines (months 3-12)
1c. Biochemical and phenotypical characterization (months 12-18)
Task 2. PTEN loss sensitizes cells to lithium in vitro. (months 18-30)
2a .Expose various isogenic pairs of PTEN knock out cell lines to lithium chloride (months 18-24)
2b. Biochemical and phenotypical characterization of response to lithium chloride (months 24-30)
Task 3. PTEN loss sensitizes cells to lithium in vivo. (months 24-36)
3a. Establish xenografts of isogenic pairs of breast cancer cell lines in nude mice (months 24-30)
3b. Treatment studies using oral and intraperitoneal administration of lithium (months 28-36)

Task 1a. Create targeting vectors (months 1-3)

The grant was awarded on June 1st 2010. Dr. Higgins graduated from her Oncology Fellowship Program in Johns Hopkins Hospital on June 30th and accepted a position as a faculty member with the Breast Cancer Program at Massachusetts General Hospital on July 1 2010. A request to transfer the grant to support Dr. Higgins as she continued this work was submitted and processed in June 2010 in anticipation of this move. Johns Hopkins University has since relinquished all interest in this grant and it was transferred to Massachusetts General Hospital in September 2010. Dr. Higgins provided a detailed progress report between June 1st 2010 and Sept 30th 2010 and a further annual report to cover the period from Sept 30th 2010 to June 30th 2011. This final report covers the period from June 1st 2010 to Sept 30 2013.

Dr. Higgins prepared and designed the planned experiments to employ gene targeting in the two breast cancer cell lines that are wild type for both *PIK3CA* and *PTEN*: HCC712 and HCC1187, which are estrogen receptor positive and negative, respectively. Targeting vectors have already been created by a former Park lab mentee (2) and were used for knocking out the *PTEN* gene in these cells.

Task 1b. Infect and screen breast epithelial and cancer cell lines (months 3-12)

Unfortunately despite several months of screening infected cells, we were unable to produce HCC712 and HCC1187 cell lines with knocked out *PTEN*. We hypothesize that this is due to the high level of genetic instability within these malignant cell lines. However; Dr. Higgins designed an alternative strategy to comprehensively study the distinct effects of several key alterations of the PI3K pathway using a library of cell lines previously created by members of the Park laboratory harboring either a *PIK3CA* mutation, (1) an *AKT1* mutation, (3) or loss of PTEN (2). Additionally Dr. Higgins and her team stably transduced Epidermal Growth Factor Receptor (EGFR) in MCF10A human breast epithelial cells using the retroviral expression vector pFBneo, which was a kind gift from Dr. Anil K. Rustgi (University of Pennsylvania). Retrovirus containing the coding sequence for EGFR was generated using Fugene6 (Roche Diagnostics, Indianapolis, IN) per the manufacturer's protocol in HEK-293T cells. Purified retrovirus was then used to infect MCF-10A cells following the manufacturer's protocol. Stable transformants were selected using 180 µg/mL G418 (Invitrogen, Carlsbad, CA). EGFR expression was confirmed by western blot using antibodies against total EGFR protein. (Figure 1) Parental MCF-10A cells were also stably transduced in parallel with an empty retroviral expression vector pFBneo (named Empty Vector or EV) and selected in the same manner to serve as controls for all experiments.

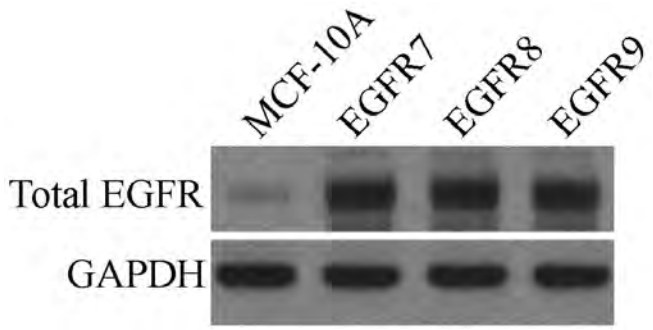


Figure 1: Overexpression of Epidermal Growth Factor Receptor in MCF-10A human breast epithelial cells. Western blot demonstrating levels of total EGFR in parental MCF-10A, and three stably transduced EGFR overexpressing clones, EGFR7, EGFR8, EGFR9. GAPDH is shown as a loading control.

Task 1c. Biochemical and phenotypical characterization (months 12-18)

Using the library of cell lines detailed above, extensive immunoblotting experiments were performed to biochemically characterize parent MCF10A cells in comparison with cell lines harboring a *PIK3CA* mutation, an *AKT1* mutation, loss of PTEN or overexpression of EGFR. We performed western blot analyses to determine the degree of MAPK and PI3K pathway activation by comparing relative levels of phosphorylated and total Akt and Erk in the absence of exogenous EGF and in the presence of physiologic concentrations of EGF.

Lysates for cells grown in each experimental condition have been prepared as previously described. (4) Western blotting was performed using the NuPage XCell SureLock electrophoresis system (Invitrogen, Carlsbad, CA) and PVDF membranes (Invitrogen, Carlsbad, CA). Primary antibodies were added overnight at 4 °C, while secondary antibodies, conjugated with horseradish peroxidase were added for 1 hr at RT. Antibodies used in this study were anti-EGFR rabbit antibody (2232; Cell Signaling Technology), anti-phospho EGFR (Tyr 1173) rabbit anti-body (4407L; Cell Signaling Technology), anti-AKT rabbit antibody (9272; Cell Signaling Technology), anti-phospho AKT (Ser 473) rabbit antibody (9271; Cell Signaling Technology), anti-p42/p44 MAP kinase rabbit antibody (9102; Cell Signaling Technology), anti-

phospho p42/p44 MAP kinase (Thr-202/Tyr-204) mouse antibody (9106; Cell Signaling Technology), anti-cyclin D1 rabbit antibody (2922; Cell Signaling Technology), anti-GSK3β rabbit antibody (9315; Cell Signaling Technology), anti-phospho GSK3β rabbit antibody (9336S; Cell Signaling Technology), and anti-GAPDH mouse antibody (6C5) (ab8245; Abcam). Blots were exposed to Kodak XAR film using chemiluminescence for detection (Perkin Elmer). All experiments were performed at least 3 times. Results of biochemical characterization of the various cell lines are shown in Figure 2.

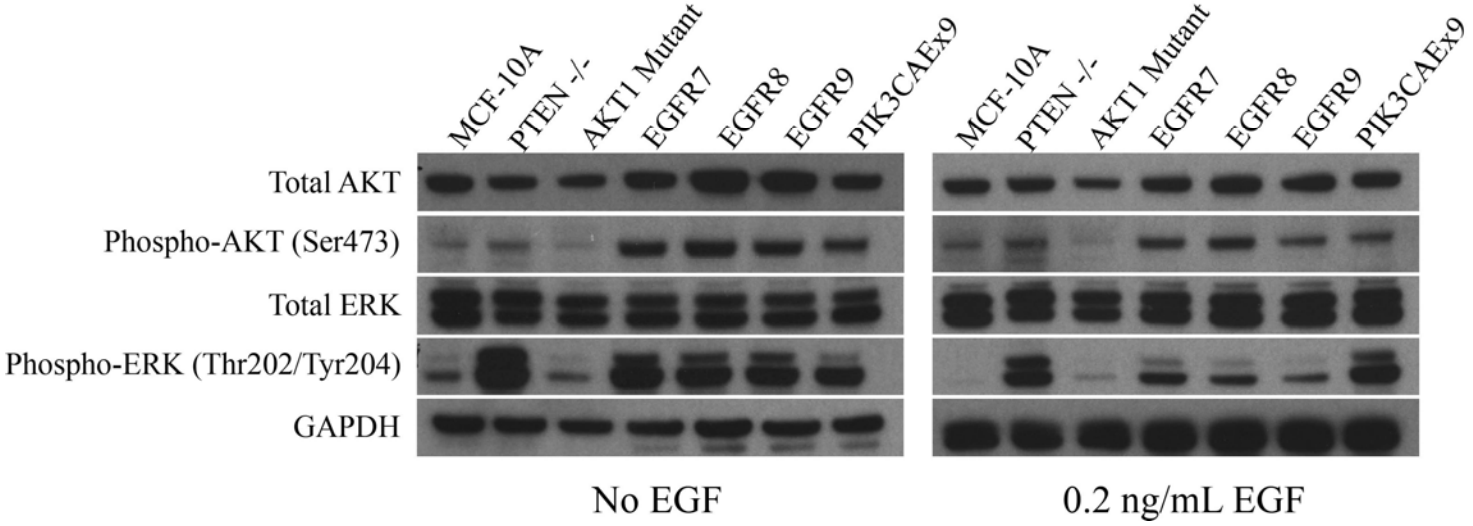


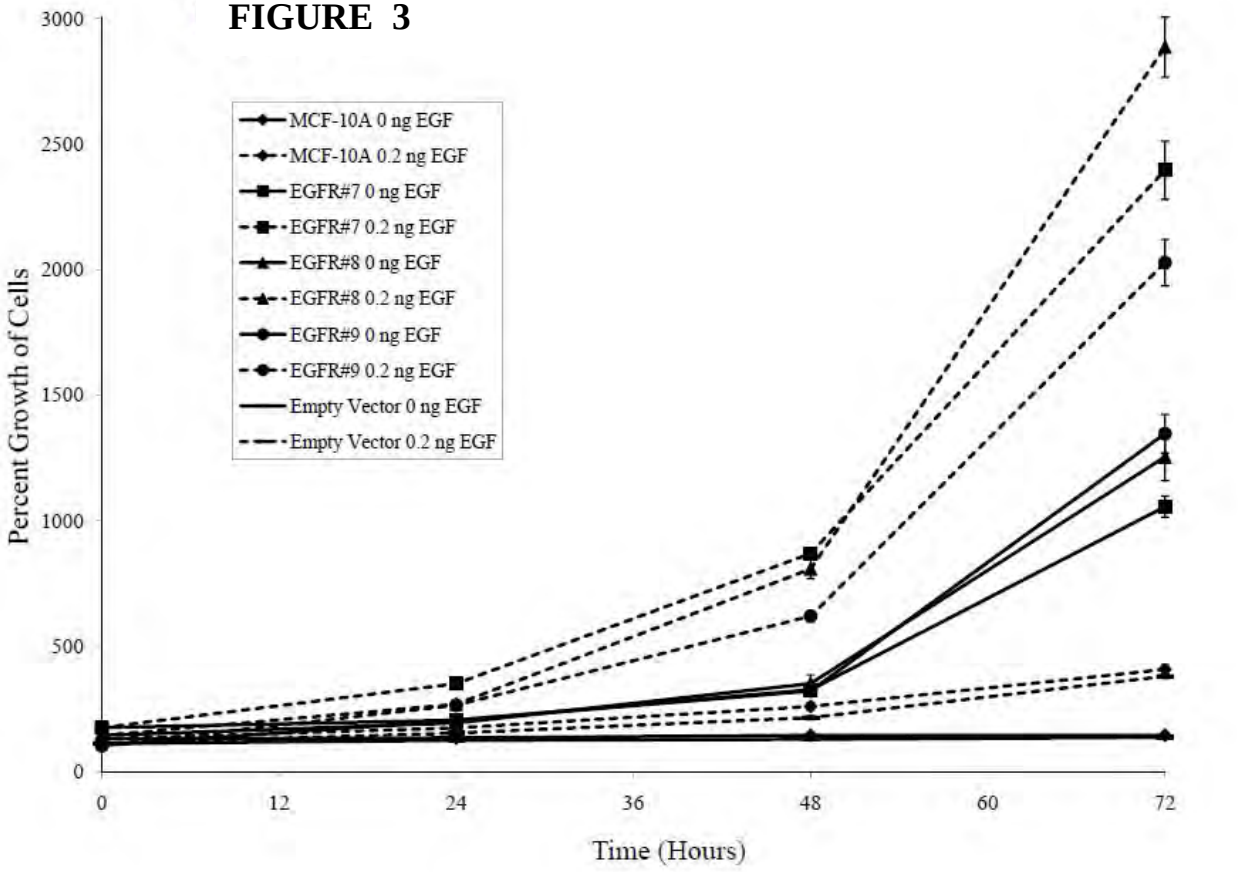
Figure 2:
Alterations in the PI3K pathway activate multiple oncogenic pathways to varying degrees. Western blot demonstrating levels of total AKT, phosphorylated AKT(Ser473), total ERK and phosphorylated ERK (Thr202/Tyr204) in parental MCF-10A, PTEN -/-, AKT1 mutant (E17K), EGFR7, EGFR8, EGFR9 (EGFR overexpressing clones) and PIK3CAEx9 (E545K) cell lines in the absence of EGF (left panel) or presence of 0.2 ng/ml EGF (right panel). GAPDH is shown as a loading control. Results are representative of multiple independent experiments.

Cell Proliferation Assays

Cells were prepared by seeding each cell line in DMEM:F12 medium without phenol red, supplemented with 1% charcoal dextran-treated fetal bovine serum (Hyclone), 10 µg/mL insulin, 0.5 µg/mL hydrocortisone, 0.1 µg/mL cholera toxin at a density of 100,000 cells per 25 cm^2. Medium was changed to either EGF-free or 0.2 ng/mL EGF-containing medium in the absence and presence of 10 mM LiCl on days 1 and 4 as indicated. Cells were counted and evaluated for viability on days 1 and 6 using a Vi-CELL Cell Viability Analyzer (Beckman Coulter). All assays and growth conditions were performed in triplicate and repeated at least 3 times.

Figure 3 - EGFR overexpression confers EGF independent growth to MCF-10A cells. Cell proliferation assays were performed as, using parental MCF-10A cells, as well as clones stably overexpressing an EGFR transgene and a control clone of MCF-10A cells stably transduced with an empty retroviral expression vector (Empty Vector). Cells were grown in the absence and presence of 0.2 ng/mL EGF (solid or dashed lines, respectively). Data points show percent growth relative to day 0 for each cell line at the displayed time. Bars represent standard error of the mean from triplicate samples. Results are representative of three independent experiments. $p < 0.001$ for all EGFR clones compared to parental MCF-10A and Empty Vector cells grown in the absence of EGF or in 0.2 ng/ml EGF.

FIGURE 3



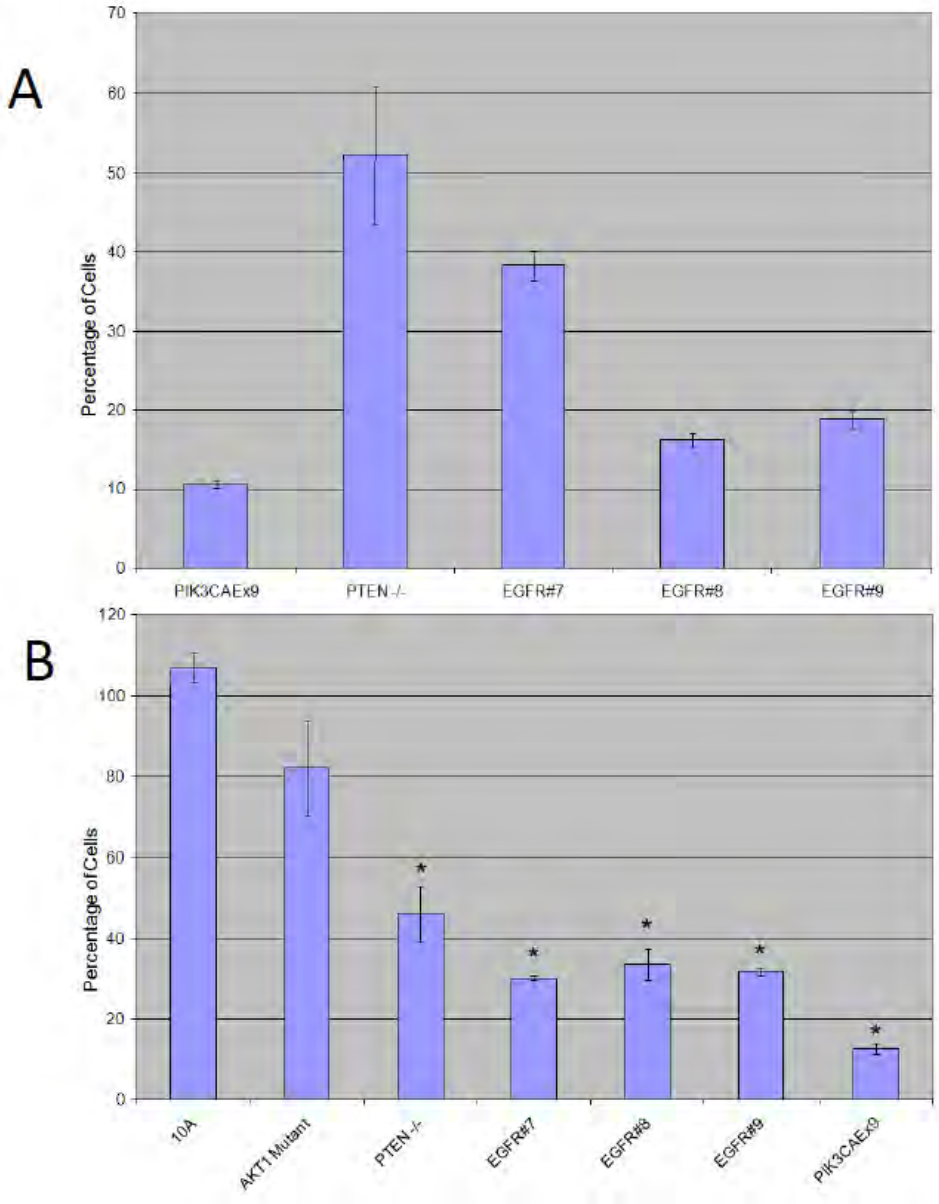
Task 2. PTEN loss sensitizes cells to lithium in vitro. (months 18-30)
2a .Expose various isogenic pairs of PTEN knock out cell lines to lithium chloride (months 18-24)

Cell proliferation assays were performed as detailed above. Standard error of the mean (SEM) was calculated for each proliferation assay. Statistical analyses were performed using a two-tailed Student's *t*-test and a one-way ANOVA across cell lines, which were calculated using Microsoft Excel and ezANOVA. A *P* value less than 0.05 was considered statistically significant.
Using identical conditions to our previous work, Dr. Higgins found that treatment with LiCl significantly inhibited the growth of cells that overexpressed EGFR, similar to the response seen with the PIK3CA knock in cell line (Fig. 4). These effects were also observed at physiologic concentrations of EGF (Fig. 4A vs. 4B).
Using a pair-wise comparison one-way ANOVA across cell lines, we found a statistically significant decrease in the proliferation of PTEN-/-, EGFR#7, EGFR#8, EGFR#9, and PIK3CAEx9 as compared with parental MCF-10A cells ($p < 0.05$). In contrast, the parental MCF-10A and AKT1 E17K cell lines were not significantly inhibited by LiCl when cultured in 0.2 ng/ml EGF ($p > 0.05$). As stated above, the effect of LiCl in parental MCF-10A and AKT1 E17K cells could not be ascertained in the absence of EGF as these cells do not proliferate under these conditions. Interestingly, the PTEN -/- cell line demonstrated intermediate sensitivity to LiCl, when compared to the response seen with PIK3CA knock in and EGFR overexpressing cell lines. Thus, although the biochemical pathways activated by mutant PIK3CA, Pten loss and EGFR overexpression appear similar, they do not uniformly predict for sensitivity to lithium treatment.

Figure 4
Lithium chloride inhibits the growth of EGFR overexpressing and *PIK3CAEx9* mutant cells but not MCF-10A parental or *AKT1* mutant cell lines. Cell proliferation and drug treatment assays were

performed with parental MCF-10A, *PTEN* -/-, *AKT1* mutant (E17K), EGFR7, EGFR8, EGFR9 (EGFR overexpressing clones) and *PIK3CAEx9* (E545K) cell lines grown in **(A)** the absence of EGF or **(B)** presence of 0.2 ng/ml EGF. Note that MCF-10A and *AKT1* mutant cells could not be included in (A) since these cells do not proliferate in the absence of EGF. Bars represent the percentage of cell proliferation in 10 mM lithium chloride *relative* to cells grown in control medium (without lithium) after 6 days in culture. Error bars represent the standard error of the mean from triplicate samples. Results are representative of three independent experiments. * $p<0.003$ compared to parental MCF-10A cells.

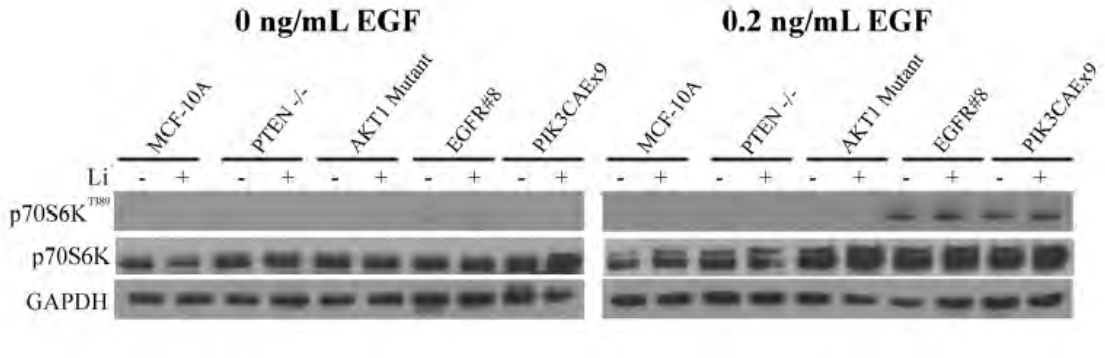
Figure 4



Task 2b. Biochemical and phenotypical characterization of response to lithium chloride (months 24-36)

To uncover the potential reasons for the differential responses to lithium seen in our panel of cell lines, we performed western blotting to elucidate any biochemical changes in the MAPK or PI3K pathways elicited by lithium exposure. Although we have previously demonstrated an increase in total GSK3β in PIK3CA knock in cells upon lithium treatment (1), this was not consistently observed in any of the EGFR overexpressing clones or PTEN -/- cells suggesting that increases in GSK3 β are not the key mediator of lithium toxicity (data not shown). In addition, we also examined levels of phosphorylated p70S6Kinase, a marker of mTOR activation, but detectable levels were only present in PIK3CA mutant cells and EGFR overexpressing clones grown in 0.2ng/ml EGF, with no appreciable change upon lithium exposure (Supplementary Fig. 2).

Supplementary Figure 2



Supplementary Figure 2 : Lithium Chloride does not affect p70S6Kinase levels or its phosphorylation.
Western blot demonstrating levels of total p70S6Kinase (p70S6K), phosphorylated p70S6Kinase ($p70S6K^{T389}$) and GAPDH (loading control) in parental MCF-10A, *PTEN-/-*, *AKT1* mutant (E17K), EGFR8, (representative EGFR overexpressing clone) and *PIK3CAEX9* (E545K) cell lines in the absence of EGF (left panel) or presence of 0.2ng/ml EGF (Right panel) in the absence (-) or presence (+) or 10mM lithium chloride (Li+). Cells were cultured and harvested at 24 hours post drug treatment as described in Materials and Methods. (5) Results are representative of multiple independent experiments.

However, consistent differences in Akt and Erk phosphorylation were seen in lithium sensitive cell lines. For example, increased phosphorylation of Akt was seen at baseline in PTEN -/-, EGFR7, EGFR8, EGFR9, and PIK3CA knock in cells and this was significantly reduced in the presence of LiCl in the EGFR overexpressing and PIK3CA knock in cells. (Fig. 5 and Supplementary Fig. 3).

Figure 5

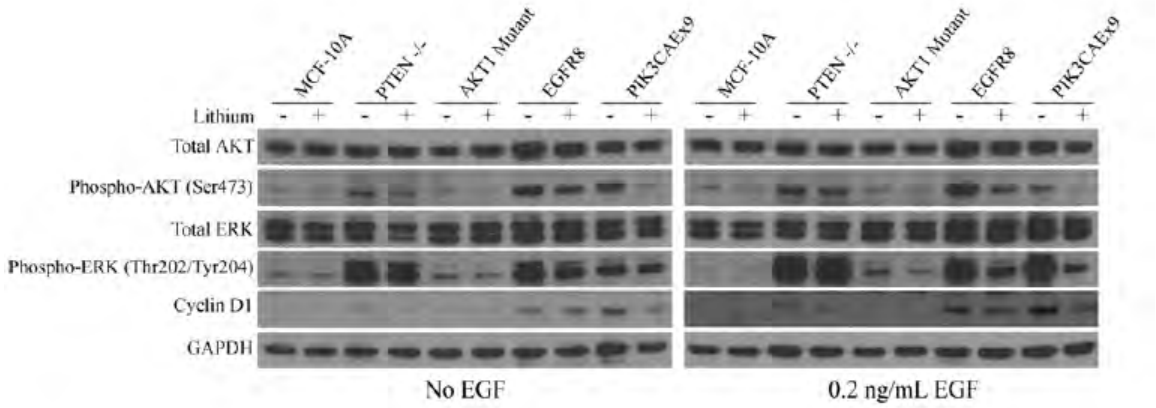
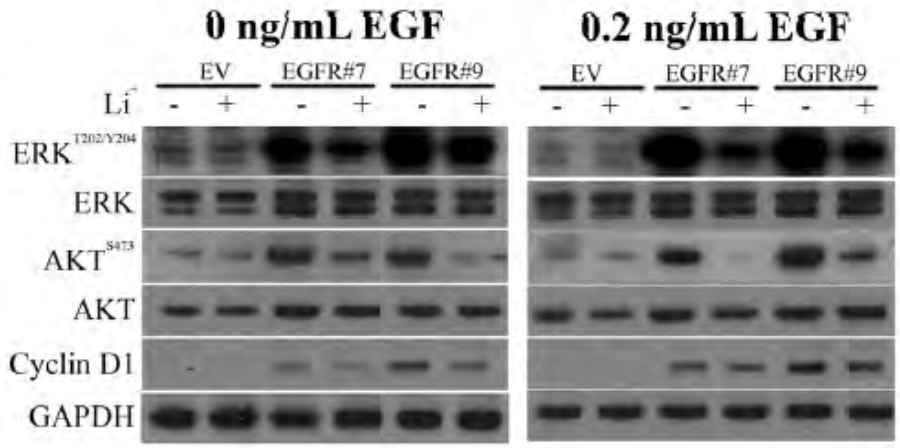


Figure 5. Mutant PIK3CAEx9 and EGFR overexpressing clones demonstrate decreased Akt and Erk phosphorylation upon lithium treatment. Western blot demonstrating levels of total AKT, phosphorylated AKT(Ser473), total ERK and phosphorylated ERK (Thr202/Tyr204) in parental MCF-10A, *PTEN -/-*, *AKT1* mutant (E17K), EGFR8, (representative EGFR overexpressing clone) and *PIK3CAEx9* (E545K) cell lines in the absence of EGF (left panel) or presence of 0.2 ng/ml EGF (right panel) in the absence (-) or presence (+) of 10 mM lithium chloride (lithium). Cells were cultured and harvested at 24 hours post drug treatment as described in Materials and Methods. (5) GAPDH is shown as a loading control. Results are representative of multiple independent experiments.

The decrease in Akt phosphorylation was far less pronounced in the PTEN -/- cells and was not appreciable in AKT1 E17K cells consistent with the response to lithium treatment observed in the growth assays. In contrast, Erk phosphorylation was slightly decreased in PTEN -/- and PIK3CA knock in cells and moderately decreased in EGFR overexpressing clones upon lithium treatment when no EGF was added to the growth medium (Fig. 5, left panel and Supplementary Fig. 3, left panel). However, marked decreases in Erk phosphorylation were seen in EGFR overexpressing cell lines and PIK3CA knock in cells when exposed to lithium under physiologic concentrations of EGF, but interestingly Erk phosphorylation appeared to be unaffected in PTEN -/- cell lines after lithium treatment under these conditions (Fig. 5, right panel). As expected, in all cases where lithium demonstrated some inhibition of proliferation, slight to moderate decreases in cyclin D1 protein were noted (Fig. 5 and Supplementary Fig. 3).

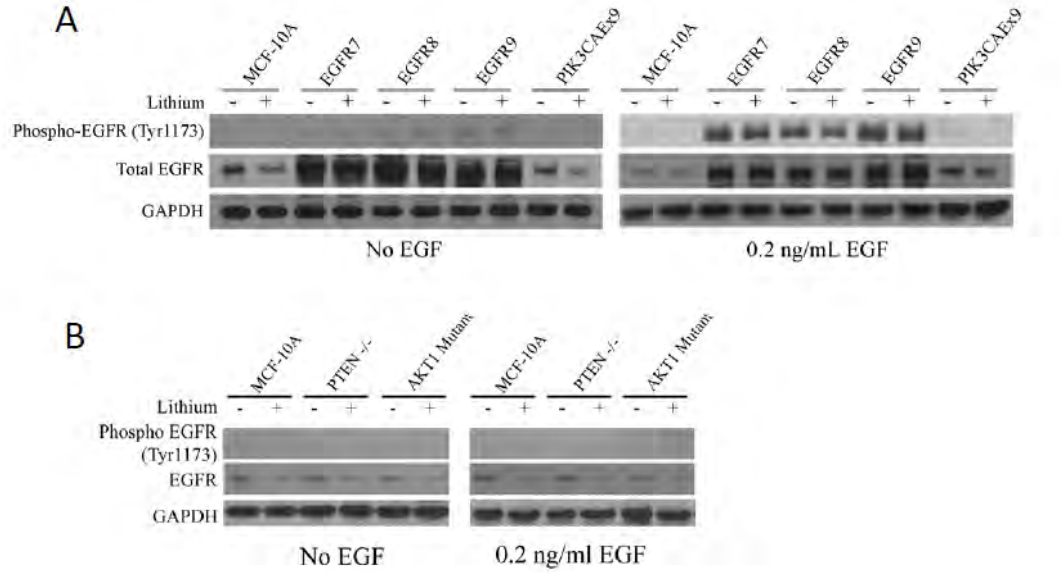
Supplementary Figure 3



Supplementary Figure 3: EGFR overexpressing clones demonstrate decreased Akt and Erk phosphorylation upon lithium treatment.
Western blot demonstrating levels of total ERK, phosphorylated ERK (Thr202/Tyr204), total AKT, phosphorylated AKT(ser473), and Cyclin D1 in Empty Vector (EV), EGFR7 and EGFR9 (EGFR overexpressing clones) cell lines in the absence of EGF (Left panel) or presence of 0.2ng/ml EGF (right panel) and in the absence (-) or presence (+) or 10mM lithium chloride (Li+). Cells were cultured and harvested at 24 hours post drug treatment as described in Materials and Methods. (5) GAPDH is shown as a loading control. Results are representative of multiple independent experiments.

The fact that lithium's effects were most pronounced on EGFR overexpressing and mutant PIK3CA knock in clones presented the intriguing possibility that perhaps lithium's effects were being mediated more proximally in the MAPK/PI3K pathways. To explore this hypothesis, we initially performed in vitro PI3K competitive kinase/ELISA assays in the presence and absence of lithium, but were unable to discern any consistent differences in PI3K in vitro activity (data not shown). We then performed western blot analyses to examine EGFR tyrosine phosphorylation (1173), a marker of EGFR activation, in the presence and absence of lithium in EGFR overexpressing, mutant PIK3CA and parental MCF-10A control cells (Supplementary Fig. 4).

Supplementary Figure 4



Supplementary Figure 4: Lithium treatment does not affect EGFR phosphorylation.
Western blot demonstrating levels of total EGFR and phosphorylated EGFR (Tyr1173) in (A) parental MCF-10A, EGFR7, EGFR8, EGFR9 (EGFR overexpressing clones), *PIK3CAEx9* (E545K) cell lines and (B) parental MCF-10A, *PTEN-/-*, and *AKT1* mutant (E18K) cell lines in the absence of EGF (Left panel) or presence of 0.2ng/ml EGF (right panel) and in the absence (-) or presence (+) or 10mM lithium chloride (Li+). Cells were cultured and harvested at 24 hours post drug treatment as described in Materials and Methods. (5) GAPDH is shown as a loading control. Results are representative of multiple independent experiments.

Interestingly, these results demonstrated that total EGFR levels decreased in parental MCF-10A,mutant PIK3CA, PTEN null and AKT1 E17K cells in the presence and absence of EGF upon lithium exposure. Although there appears to also be decreased EGFR phosphorylation with lithium in EGF containing conditions, this is likely due to total EGFR levels decreasing. EGFR overexpressing clones however, did not show any appreciable change in total or phosphorylated EGFR with lithium treatment regardless of whether EGF was present in the media. However, due to the fact that decreases in total EGFR did not correlate with sensitivity to lithium, it can be concluded that changes in total EGFR protein or its phosphorylation are not responsible for the growth inhibitory effects of lithium in mutant PIK3CA and EGFR overexpressing cell lines.

Task 3. PTEN loss sensitizes cells to lithium in vivo. (months 24-36)
3a. Establish xenografts of isogenic pairs of breast cancer cell lines in nude mice (months 24-30)
3b. Treatment studies using oral and intraperitoneal administration of lithium (months 28-36)

As outlined in the revised statement of work provided in November 2010, there was a gap in funding during the first months of this work and the treatment studies planned for months 28-36 were not completed in their entirety. The biochemical and phenotypical characterization of response to lithium chloride in fact consumed most of the time period from months 24-36.
Since the design of this project over 5 years ago, other PI3K inhibitors have been developed and have progressed through phase I and II human clinical trial development. These compounds appear to have an improved therapeutic index over lithium therefore we no longer feel that in vivo assessment of lithium will be relevant. Our group has instead focused on novel techniques to identify patients whose tumors possess somatic *PIK3CA* mutations as these are the patients who should be considered for trials of PI3K inhibitors. During months 24-36 of this grant period we have published our complementary work in this field also. (6)

Key research accomplishments

1. Overexpression of Epidermal Growth Factor Receptor in MCF-10A human breast epithelial cells
2. Collation of a library of cell lines each representative of distinct genetic alterations of the PI3K/AKT/mTOR pathway
3. Biochemical and phenotypical characterization of the above cell lines is complete
4. Phenotypical characterization of the above cell lines' response to lithium chloride.
5. Biochemical characterization of the above cell lines' response to lithium chloride.

Reportable Outcomes

- The results to date were presented at the Department of Defense Era of Hope Conference, Florida, August 2011 as a poster presentation.
- Results were published in Cancer Biology & Therapy, February 2011 (PDF included in appendix)
- Original manuscript was editorialized in Cancer biology & Therapy, February 2011 (PDF included in appendix)
- Follow-on project was published in Clinical Cancer Research, March 2012 (PDF included in appendix)

Conclusions

PIK3CA mutations, EGFR overexpression, Pten loss and *AKT1* E17K mutations have different biochemical signaling profiles and respond differently to pharmacologic treatment with Lithium Chloride.

Cell-based models of human cancers have traditionally been used for drug discovery. Though their use has led to the development of effective therapies, lack of proper control cells has hindered the ability to validate a given compound's true target or mechanism of action and has often led to disappointing results in clinical trials. For example, the small molecule inhibitors gefitinib and erlotinib were originally proposed to be effective agents against lung cancer cells overexpressing EGFR although only 10% of patients responded to these drugs. Retrospective examination of these tumors demonstrated that certain EGFR mutations were associated with responses (7-9), thus these mutations, rather than EGFR overexpression, have become better predictors of response to gefitinib and erlotinib (10). Recently, the use of isogenic non-tumorigenic human breast epithelial cell lines has been proposed as a model for drug discovery using targeted gene replacement strategies (11). Indeed, these studies demonstrated that EGFR mutations knocked into human breast epithelial cells dramatically sensitized cells to erlotinib thus recapitulating human clinical trial data. Additionally, prospective proof of principle concepts using isogenic cell lines to develop cancer therapies have been realized. A recent exciting clinical trial demonstrated the efficacy of poly (ADP-ribose) polymerase (PARP) inhibitors in BRCA mutation positive breast cancers (12). This was the direct result of a "synthetic lethal" drug screen employing isogenic cell lines to identify PARP inhibitors' selectivity for BRCA null cells (13,14). The data from these clinical trials demonstrate that better defined genetic models of cancer can ultimately result in highly effective targeted therapies and equally important, reliable predictors of response to those therapies.

Uncontrolled activity of the PI3K signaling pathway is found in many cancers. Our study further characterizes the disparate effects of various aberrations that occur within the PI3K pathway in human breast cancers using our unique library of isogenic cell lines. Loss of the tumor suppressor Pten is common in breast cancers (15,16). We verify here that such loss is associated with increased constitutive activation of the MAPK and PI3K pathways; however, the level of Akt activation is not as extensive as that caused by a *PIK3CA* mutation and does not confer equivalent sensitivity to the toxic effects of lithium. In contrast to *PIK3CA* mutations and Pten loss, the presence of an isolated *AKT1* E17K mutation leads to minimal changes in the MAPK and PI3K pathways and as such, these cells appear relatively resistant to lithium compared to cells harboring an oncogenic *PIK3CA* mutation.

We demonstrate in this study that overexpression of EGFR leads to activation of multiple oncogenic pathways in a similar manner as somatic cell knock in of mutant *PIK3CA*. Thus a mutation of PIK3CA appears to mimic a proximal alteration resulting in the complex activation of multiple pathways including PI3K and MAPK signaling. Although activation of these two pathways by EGFR overexpression was expected based upon previous knowledge of RTK signaling, the signaling induced by mutant *PIK3CA*

is confirmed to be of greater complexity as we previously reported (1). We hypothesize that "rewiring" by mutant PIK3CA leads to MAPK pathway activation, perhaps by virtue of the known binding that occurs via Ras and the Ras binding domain of p110α. Our results demonstrate that targeted therapies against key components in both the MAPK and PI3K pathways may yield the most effective results in cancers that harbor *PIK3CA* mutations. This may be especially relevant given the current inability to isolate mutant specific PI3K inhibitors. Interestingly, various inhibitors of the PI3K and MAP Kinase pathways such as wortmannin, LY294002, U0126 and rapamycin have shown some activity in our *PIK3CA* knock in and PTEN knock out cell lines 15, 25, 26, but depending on the culture conditions, non-selective toxicity due to off target effects was also present making interpretation of dual pathway inhibition difficult to assess.

Interestingly, loss of Pten led to only a partial sensitivity to lithium treatment despite baseline increases in Akt and Erk phosphorylation. However, in the absence of exogenous EGF, PTEN knock out cells had relatively less Akt phosphorylation and relatively more Erk phosphorylation compared to the highly lithium sensitive EGFR overexpressing and mutant *PIK3CA* cell lines. Intriguingly, lithium treatment of PTEN -/- cells modestly decreased Akt phosphorylation, yet there was no appreciable effect on Erk phosphorylation under physiologic EGF concentrations, a result that was distinct from EGFR overexpressing and mutant *PIK3CA* clones. Because of the isogenic nature of these cell lines, our results provide further evidence against the notion that pathway alterations in the PI3K pathway are functionally similar. Parsons and colleagues reported the newly discovered role of P-Rex2a in regulating Pten activity, as well as increased P-Rex2a activity in cancers with PIK3CA mutations (17). It is tempting to speculate that changes in P-Rex2a activity may account for some of the differences seen in our isogenic system between PTEN -/- and mutant PIK3CA knock in cell lines.

Lithium, an FDA approved drug, is classically thought to be an inhibitor of GSK3β, but paradoxically, decreased tumor growth has been seen in many systems using lithium and other GSK3β inhibitors (18-22). In our previous studies, we demonstrated that lithium decreases epithelial cell proliferation in cells harboring mutant PIK3CA and this was correlated with the upregulation of GSK3β, a known growth suppressor. However, in the current study increases in GSK3β were not seen in EGFR overexpressing clones despite the fact that these cell lines were otherwise phenotypically identical to mutant *PIK3CA* knock in cells (data not shown). More surprising was the finding that Akt and Erk, two molecular mediators of GSK3β phosphorylation and its subsequent inactivation (21, 23, 24), demonstrated significantly decreased phosphorylation in EGFR overexpressing and mutant *PIK3CA* knock in cell lines upon lithium treatment. We conclude that significant activation of both PI3K and MAPK pathways beyond a critical threshold may be required for lithium sensitivity. These results suggest that lithium's mechanism of action may not be at the level of GSK3β, but rather more proximal in the signaling cascades induced by these oncogenic stimuli. We therefore hypothesized that lithium may affect more upstream events such as PI3K activity or phosphorylation of the EGFR itself. However, our analyses excluded these possibilities. The fact that Pten null cells displayed only partial sensitivity to lithium and did not display decreases in Erk phosphorylation with lithium treatment suggests once again that loss of Pten is functionally distinct from mutant *PIK3CA*. Equally important, because our experiments were performed in isogenic cell lines, direct and conclusive comparison of different genetic alterations within the same pathway can be made. Thus, our study suggests that the somatic changes that occur in human cancers may not be used interchangeably as predictors of response to a given pathway inhibitor as evidenced by our varying results with lithium sensitivity.

So what?

Our results have significant potential for clinical relevance beyond the ability to use isogenic cell lines to validate targets of therapy and predictive markers of response. Based on the experiments presented here, there is the possibility of using this information clinically for the treatment of breast cancer patients whose tumors harbor mutant *PIK3CA* and/or EGFR overexpression. For example, combination therapy with lithium may be a strategy to prevent drug resistance, since previous studies have noted that EGFR expression is associated with resistance to hormonal and chemotherapies in breast cancers (25). In addition, the majority of breast tumors harboring a *PIK3CA* mutation are also hormone receptor positive, yet activation of the PI3K pathway is recognized as a mechanism of endocrine resistance (26), and resistance to HER2-targeted agents (27,28). Thus we envision that the addition of lithium therapy to current chemo, hormone and HER2 directed therapies may augment the clinical efficacy of these agents and perhaps reduce the emergence of drug resistant tumors.

References

1. Gustin JP, Karakas B, Weiss MB, Abukhdeir AM, Lauring J, Garay JP, et al. Knockin of mutant PIK3CA activates multiple oncogenic pathways. Proc Natl Acad Sci U S A. 2009;106:2835-40.
2. Vitolo MI, Weiss MB, Szmacinski M, Tahir K, Waldman T, Park BH, et al. Deletion of PTEN Promotes Tumorigenic Signaling, Resistance to Anoikis, and Altered Response to Chemotherapeutic Agents in Human Mammary Epithelial Cells. Cancer Res. 2009.
3. Lauring J, Cosgrove DP, Fontana S, Gustin JP, Konishi H, Abukhdeir AM, et al. Knock in of the AKT1 E17K mutation in human breast epithelial cells does not recapitulate oncogenic PIK3CA mutations. Oncogene. 2010.
4. Konishi H, Karakas B, Abukhdeir AM, Lauring J, Gustin JP, Garay JP, et al. Knock-in of mutant K-ras in nontumorigenic human epithelial cells as a new model for studying K-ras mediated transformation. Cancer Res. 2007;67:8460-7.
5. Higgins MJ, Beaver JA, Wong HY, Gustin JP, Lauring JD, Garay JP, et al. PIK3CA mutations and EGFR overexpression predict for lithium sensitivity in human breast epithelial cells. Cancer Biol and Ther. 2011;11:3, 358-367
6. Higgins MJ, Jelovac D, Barnathan E, Blair B, Slater S, Powers P, et al. Detection of tumor PIK3CA status in metastatic breast cancer using peripheral blood. Clin Cancer Res. 2012;15;18(12):3462-9
7. Lynch TJ, Bell DW, Sordella R, Gurubhagavatula S, Okimoto RA, Brannigan BW, et al. Activating mutations in the epidermal growth factor receptor underlying responsiveness of non-small-cell lung cancer to gefitinib. N Engl J Med 2004; 350:2129-39
8. Paez JG, Janne PA, Lee JC, Tracy S, Greulich H, Gabriel S, et al. EGFR mutations in lung cancer: correlation with clinical response to gefitinib therapy. Science 2004; 304:1497-500
9. Pao W, Miller V, Zakowski M, Doherty J, Politi K, Sarkaria, J et al. EGF receptor gene mutatiosn are comon in lung cancers from 'never smokers' and are associated with sensitivity of tumors to gefitinib and erlotinib. Proc natl Acad Sci USA 2004; 101:13306-11
10. Rosell R, Moran T, Queralt C, Porta R, Cardenal F, Camps C, et al. Screening for epidermal growth factor receptor mutations in lung cancer. N Engl J Med 2009; 361:958-67.
11. Di Nicolantonio F, Arena S, Gallicchio M, Zecchin D, Martini M, Flonta SE, et al. Replacement of normal with mutant alleles in the genome of normal human cells unveils mutation-specific drug responses. Proc natl Acad Sci USA 2008; 105:20864-9
12. Fong PC, Boss DS, Yap TA, Tutt A, Wu P, Mergui-Roelvink M, et al. Inhibition of poly(ADP-ribose) polymerase in tumors from BRCA mutation carriers. N Engl J Med 2009; 361:123-34.
13. Farmer H, McCabe N, Lord CJ, Tutt AN, Johnson DA, Richardson TB, et al. Targeting the DNA repair defect in BRCA mutant cells as a therapeutic strategy. Nature 2005; 434:917-21.
14. Bryant HE, Schultz N, Thomas HD, Parker KM, Flower D, Lopez E, et al. Specific killing of BRCA2- deficient tumours with inhibitors of poly(ADP-ribose) polymerase. Nature 2005; 434:913-7.
15. Depowski PL, Rosenthal SI, Ross JS. Loss of expression of the PTEN gene protein product is associated with poor outcome in breast cancer. Mod Pathol 2001; 14:672-6.
16. Perren A, Weng LP, Boag AH, Ziebold U, Thakore K, Dahia PL, et al. Immunohistochemical evidence of loss of PTEN expression in primary ductal adenocarcinomas of the breast. Am J Pathol 1999; 155:1253-60.
17. Fine B, Hodakoski C, Koujak S, Su T, Saal LH, Maurer M, et al. Activation of the PI3K pathway in cancer through inhibition of PTEN by exchange factor P-REX2a. Science 2009; 325:1261-5.
18. Wang Z, Smith KS, Murphy M, Piloto O, Somervaille TC, Cleary ML. Glycogen synthase kinase 3 in MLL leukaemia maintenance and targeted therapy. Nature. 2008.
19. Korur S, Huber RM, Sivasankaran B, Petrich M, Morin P, Jr., Hemmings BA, et al. GSK3beta regulates differentiation and growth arrest in glioblastoma. PLoS One. 2009;4:e7443.
20. Beurel E, Blivet-Van Eggelpoel MJ, Kornprobst M, Moritz S, Delelo R, Paye F, et al. Glycogen synthase kinase-3 inhibitors augment TRAIL-induced apoptotic death in human hepatoma cells. Biochem Pharmacol. 2009;77:54-65.
21. Kunnimalaiyaan M, Vaccaro AM, Ndiaye MA, Chen H. Inactivation of glycogen synthase kinase-3beta, a downstream target of the raf-1 pathway, is associated with growth suppression in medullary thyroid cancer cells. Mol Cancer Ther. 2007;6:1151-8.
22. Cao Q, Lu X, Feng YJ. Glycogen synthase kinase-3beta positively regulates the proliferation of human ovarian cancer cells. Cell research. 2006;16:671-7.

23. Cross DA, Alessi DR, Cohen P, Andjelkovich M, Hemmings BA. Inhibition of glycogen synthase kinase-3 by insulin mediated by protein kinase B. Nature 1995; 378:785-9.
24. Ding Q, Xia W, Liu JC, Yang JY, Lee DF, Xia J, et al. Erk associates with and primes GSK-3beta for its inactivation resulting in upregulation of beta-catenin. Mol Cell 2005; 19:159-70
25. Arpino G, Green SJ, Allred DC, Lew D, Martino S, Osborne CK, et al. HER-2 amplification, HER-1 expression and tamoxifen response in estrogen receptor- positive metastatic breast cancer: a southwest oncology group study. Clin Cancer Res 2004; 10:5670-6.
26. Campbell RA, Bhat-Kakshatri P, Patel NM, Constantinidou D, Ali S, Nakshatri H. Phosphatidylinositol 3-kinase/AKT-mediated activation of estrogen receptor alpha: a new model for antiestrogen resistance. J Biol Chem 2001; 276:9817-24.
27. Nagata Y, Lan KH, Zhou X, Tan M, Esteva FJ, Sahin AA, et al. PTEN activation contributes to tumor inhibition by trastuzumab, and loss of PTEN predicts trastuzumab resistance in patients. Cancer Cell 2004; 6:117-27
28. Berns K, Horlings HM, Hennessy BT, Madiredjo M, Hijmans EM, Beelen K, et al. A functional genetic approach identifies the PI3K pathway as a major determinant of trastuzumab resistance in breast cancer. Cancer Cell 2007; 12:395-402.

Appendices

Cancer Biology & Therapy 11:3, 358-367; February 1, 2011; © 2011 Landes Bioscience

PIK3CA mutations and EGFR overexpression predict for lithium sensitivity in human breast epithelial cells

Michaela J. Higgins, Julia A. Beaver, Hong Yuen Wong, John P. Gustin, Josh D. Lauring, Joseph P. Garay, Hiroyuki Konishi, Morassa Mohseni, Grace M. Wang, Justin Cidado, Danijela Jelovac, David P. Cosgrove, Akina Tamaki, Abde M. Abukhdeir* and Ben Ho Park*

The Sidney Kimmel Comprehensive Cancer Center; The Johns Hopkins University School of Medicine; Baltimore, MD USA

Key words: breast cancer, AKT, PI3K, Pten, EGFR

Abbreviations: EGF, epidermal growth factor; EGFR, epidermal growth factor receptor; mTOR, mammalian target of rapamycin; Pten, phosphatase and tensin homolog; PI3K, phosphatidylinositol 3-kinase

A high frequency of somatic mutations has been found in breast cancers within the gene encoding the catalytic p110α subunit of PI3K, *PIK3CA*. Using isogenic human breast epithelial cells, we have previously demonstrated that oncogenic *PIK3CA* "hotspot" mutations predict for response to the toxic effects of lithium. However, other somatic genetic alterations occur within this pathway in breast cancers, and it is possible that these changes may also predict for lithium sensitivity. We overexpressed the epidermal growth factor receptor (EGFR) into the non-tumorigenic human breast epithelial cell line MCF-10A, and compared these cells to isogenic cell lines previously created via somatic cell gene targeting to model Pten loss, *PIK3CA* mutations, and the invariant *AKT1* mutation, E17K. EGFR overexpressing clones were capable of cellular proliferation in the absence of EGF and were sensitive to lithium similar to the results previously seen with cells harboring *PIK3CA* mutations. In contrast, *AKT1* E17K cells and *PTEN*$^{-/-}$ cells displayed resistance or partial sensitivity to lithium, respectively. Western blot analysis demonstrated that lithium sensitivity correlated with significant decreases in both PI3K and MAPK signaling that were observed only in EGFR overexpressing and mutant *PIK3CA* cell lines. These studies demonstrate that EGFR overexpression and *PIK3CA* mutations are predictors of response to lithium, whereas Pten loss and *AKT1* E17K mutations do not predict for lithium sensitivity. Our findings may have important implications for the use of these genetic lesions in breast cancer patients as predictive markers of response to emerging PI3K pathway inhibitors.

Introduction

The phosphatidylinositol 3-kinase (PI3K) pathway regulates many important cellular processes including angiogenesis, proliferation and apoptosis.[1] The catalytic and regulatory subunits of the human *PIK3CA* gene were cloned over fifteen years ago[2] and somatic mutations in the gene encoding for the PI3K p110α catalytic subunit, *PIK3CA*, have subsequently been identified in many cancers (reviewed in ref. 3). The frequency of *PIK3CA* mutations in human breast cancers ranges in studies from 8–40%[4-7] with an average of 25%, an observation that supports the significance of PI3K in breast cancer biology and underscores its importance as a potential therapeutic target.

Additional somatic alterations are also found in key genes that lie within the PI3K pathway. For example, the phosphatase and tensin homolog (Pten) protein is a tumor suppressor that reverses the effects of PI3K by dephosphorylating the 3' phosphate of the inositol ring in phosphatidylinositol-(3,4,5)-trisphosphate resulting in phosphatidylinositol-(4,5)-bisphosphate. Although rarely mutated in breast cancer, diminished levels of Pten expression through loss of heterozygosity and/or epigenetic silencing mechanisms are observed in up to 48% of tumors.[8,9] Furthermore, aberrant Pten activity in breast cancers has been associated with metastasis and poor survival.[8,10] Another critical member of the PI3K pathway is Akt. Akt family members are frequently activated in cancers via phosphorylation. Recently a single hotspot mutation, G49A:E17K, in the pleckstrin homology domain of *AKT1* has been described, with the highest frequency of mutations found in human breast cancers.[11] Subsequent studies have confirmed the relatively low but consistent frequency of this mutation ranging from 1.8–8%.[12-15]

Aberrant activation of the PI3K pathway in breast cancers also occurs through the human erbB receptor tyrosine kinase (RTK) family of transmembrane receptors which includes epidermal growth factor receptor (EGFR), HER2, HER3 and HER4. Although RTK activation leads to MAPK signaling via Ras, Raf,

*Correspondence to: Abde M. Abukhdeir and Ben Ho Park; Email: aabukhd@hotmail.com and bpark2@jhmi.edu
Submitted: 11/05/10; Accepted: 11/18/10
DOI: 10.4161/cbt.11.3.14227

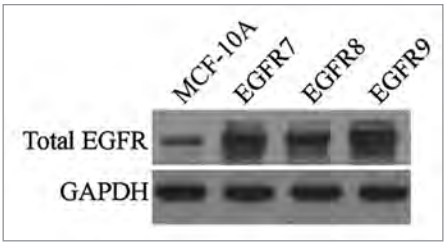


Figure 1. Overexpression of Epidermal Growth Factor Receptor in MCF-10A human breast epithelial cells. Western blot demonstrating levels of total EGFR in parental MCF-10A, and three stably transduced EGFR overexpressing clones, EGFR7, EGFR8, EGFR9. GAPDH is shown as a loading control. Results are representative of three independent experiments.

Mek and Erk, it is now known that RTK activation also results in signaling through the PI3K pathway via Ras/p110α binding as well as through the intermediate molecule IRS-1.[16,17] EGFR overexpression has been reported in breast cancers and is associated with resistance to hormonal therapy and reduced disease free survival.[18-20] In addition, approximately 40% of triple negative/basal type breast cancers are associated with overexpression of EGFR. Moreover, HER2 and concurrent EGFR expression is found in 21% of breast tumors.[21] EGFR is therefore an attractive target in breast cancer, but to date clinical trials of single agent tyrosine kinase inhibitors have been disappointing.[22] Successful translation for benefit will require a better understanding of the complex pathways involved with EGFR signaling leading to novel combinations of cytotoxic therapies.[23]

We and others have previously created physiologic in vitro models of aberrant oncogenic PI3K pathway signaling by employing somatic cell gene targeting in the human breast epithelial cell line, MCF-10A.[24-27] MCF-10A cells are spontaneously immortalized cells and provide an ideal model for these experiments because they are human, mostly diploid, non-tumorigenic and are genetically stable by FISH,[28] karyotype,[29] copy number variation and microsatellite analyses (data not shown). The use of paired isogenic cell lines provides a unique opportunity to study the effects of oncogenic *PIK3CA* mutations on downstream signaling pathways with less concern about potential confounding genetic anomalies. Consequently, we determined that lithium, an FDA-approved therapy for bipolar disorder, has selective anti-neoplastic properties against human breast and colon cancer cell lines that harbor oncogenic *PIK3CA* mutations.[25] However, other genetic alterations in the PI3K pathway such as Pten loss, *AKT1* E17K mutations and EGFR overexpression occur in human breast cancers. Although previous work has suggested that mutations in genes within a common pathway are functionally equivalent and, therefore, rarely occur concurrently in human malignancies, this notion has been recently challenged.[13] Indeed, our own previous studies have demonstrated dramatic phenotypic differences between *PIK3CA* and *AKT1* mutations.[15] It therefore remains an open question as to whether genetic lesions in the same pathway will be equivalent in their ability to predict for response to a given pathway inhibitor. Therefore, we sought to determine if these genetic alterations would also recapitulate equivalent lithium sensitivity by employing our isogenic cell based system. Using isogenic MCF-10A derived *PTEN* knock out cells (*PTEN*$^{-/-}$),[26] *AKT1* E17K knock in cells (AKT1 mutant)[15] and EGFR overexpressing cells, we determined that only EGFR overexpression exhibited a similar signal transduction pattern and sensitivity to lithium similar to mutant *PIK3CA* knock in cell lines. This work has potential implications for the development of predictive biomarkers of response to future targeted therapies.

Results

Characterization of EGFR overexpressing MCF-10A cell lines. Based upon our previous study in reference 25, oncogenic *PIK3CA* mutations appear to activate both MAPK and PI3K pathways in a manner akin to erbB receptor activation. This led us to hypothesize that EGFR overexpression, which is present in a significant fraction of triple negative breast cancers, may also result in lithium sensitivity. To effectively model this, we chose to overexpress a human EGFR cDNA in the non-tumorigenic breast epithelial cell line, MCF-10A. Using a retroviral infection strategy, we were able to isolate three EGFR-overexpressing clones, which were named EGFR7, EGFR8 and EGFR9. Furthermore, we also isolated a G418-resistant, non-EGFR overexpressing control clone transduced with an empty vector (EV). Overexpression of EGFR protein was confirmed by western blot using antibodies against total EGFR protein (**Fig. 1**).

EGFR overexpression is thought to lead to homodimerization of EGFR as well as heterodimerization with other erbB family members resulting in autophosphorylation and activation of these RTKs. Because MCF-10A cells require exogenous EGF for cellular proliferation, we tested the EGFR overexpressing clones for their ability to proliferate under EGF free conditions as well as physiologic concentrations of EGF (0.2 ng/ml) as previously described in reference 25. **Figure 2** displays the proliferation of parental MCF-10A cells, our three independently-derived EGFR overexpressing clones, and an empty vector control in the absence and presence of 0.2 ng/mL EGF. All three of our EGFR overexpressing clones were capable of statistically-significant, EGF-independent proliferation, compared to control cells as measured after 72 hours by Student's t-test ($p < 0.05$). In the presence of EGF, the increased rate of proliferation was also statistically significant ($p < 0.05$). The empty vector clone was not capable of EGF-independent growth and it's growth in the presence of EGF was not statistically different from parental MCF-10A cells ($p > 0.05$). These EGF independent growth properties were similar to what we have previously described for PIK3CA knock in mutant and *PTEN* knock out MCF-10A derived cell lines.[25,26] Although theoretically it is possible that EGF independent growth was the result of increased EGF ligands secreted by these genetically modified cell lines, western blot analysis and co-culture using transwell assays did not support this hypothesis (**Sup. Fig. 1**).

***PIK3CA* mutations, EGFR overexpression, Pten loss and *AKT1* E17K mutations have different biochemical signaling profiles.** Although *PIK3CA* mutations, Pten loss and *AKT1* E17K mutations were originally thought to be functionally

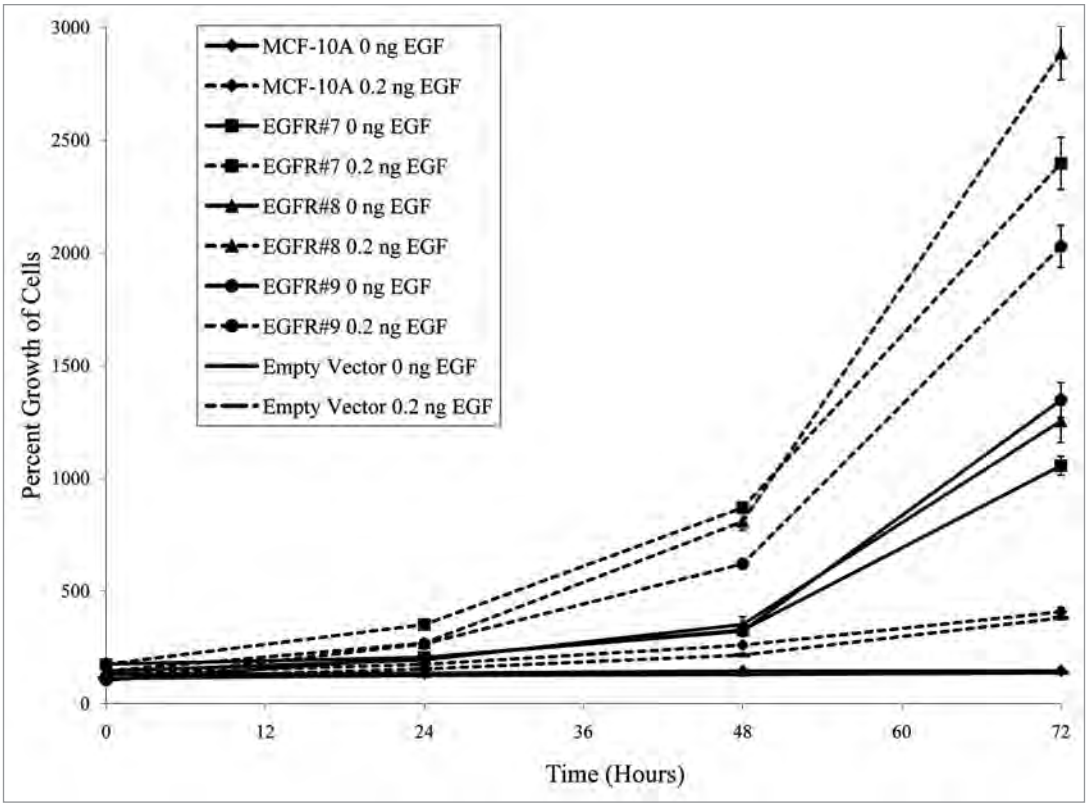


Figure 2. EGFR overexpression confers EGF independent growth to MCF-10A cells. Cell proliferation assays were performed as described in Materials and Methods, using parental MCF-10A cells, as well as clones stably overexpressing an EGFR transgene and a control clone of MCF-10A cells stably transduced with an empty retroviral expression vector (Empty Vector). Cells were grown in the absence and presence of 0.2 ng/mL EGF (solid or dashed lines, respectively). Data points show percent growth relative to day 0 for each cell line at the displayed time. Bars represent standard error of the mean from triplicate samples. Results are representative of three independent experiments. $p < 0.001$ for all EGFR clones compared to parental MCF-10A and Empty Vector cells grown in the absence of EGF or in 0.2 ng/ml EGF.

equivalent based upon their mutual exclusivity in human cancers,[7,11] recent studies have now demonstrated differences in pathway activation among these genetic alterations as well as rare cancers that have lost Pten and concurrently harbor an activating *PIK3CA* mutation.[13,30] In order to perform comparative analyses between *PIK3CA* knock in, *PTEN* knock out, *AKT1* E17K knock in and EGFR overexpressing cells in the same MCF-10A background, we first performed Western blot analyses to determine the degree of MAPK and PI3K pathway activation by comparing relative levels of phosphorylated and total Akt and Erk in the absence of exogenous EGF and in the presence of physiologic concentrations of EGF (**Fig. 3**). The rationale for examining our cell lines in the absence and presence of EGF stems from our previous work demonstrating that EGF exposure at varying levels can lead to dramatic differences in biochemical signaling as well as response to drugs such as the mTOR inhibitor, rapamycin.[25] It should be noted that blots were performed separately for no EGF versus 0.2 ng/ml EGF conditions, such that comparisons can only be made between cell lines within each EGF culture condition. Direct comparisons of signaling pathways between no EGF versus 0.2 ng/ml of EGF have been previously described for PIK3CA mutant, *PTEN* knock out and AKT1 E17K cell lines.[15,25,26]

Representative clones for *PIK3CA* exon 9 knock in, *PTEN* knock out and *AKT1* E17K knock in were used as these cell lines have all previously been described to be indistinct from their clonal sibs.[15,25,26] Because EGFR overexpressing clones have not yet been characterized, all three clones were used for these studies. Previously, we have described that *AKT1* E17K knock in cells do not proliferate in the absence of EGF and concordantly, they display minimal phosphorylation of Akt and Erk.[15] As seen in **Figure 3**, *AKT1* E17K cells did not demonstrate any significant activation of the PI3K or MAPK pathways relative to parental MCF-10A cells, as shown by the minimal phosphorylation of Akt and Erk under EGF free and physiologic concentrations of EGF (0.2 ng/ml). However, in the presence of EGF, there was a slight but reproducible increase in ERK phosphorylation in *AKT1* E17K cells relative to parental MCF-10A cells (**Fig. 3** and right panel). The reason for this is unclear, but reaffirms the notion that signaling via EGF/EGFR can lead to unexpected and varying responses depending on the genetic alterations present within a given cell. In contrast, phosphorylated Akt was increased in the *PTEN*$^{-/-}$ cell line, but this was not as pronounced as in the three EGFR overexpressing clones or the *PIK3CA* knock in cell line in conditions without exogenous EGF (**Fig. 3** and left panel), though was comparable

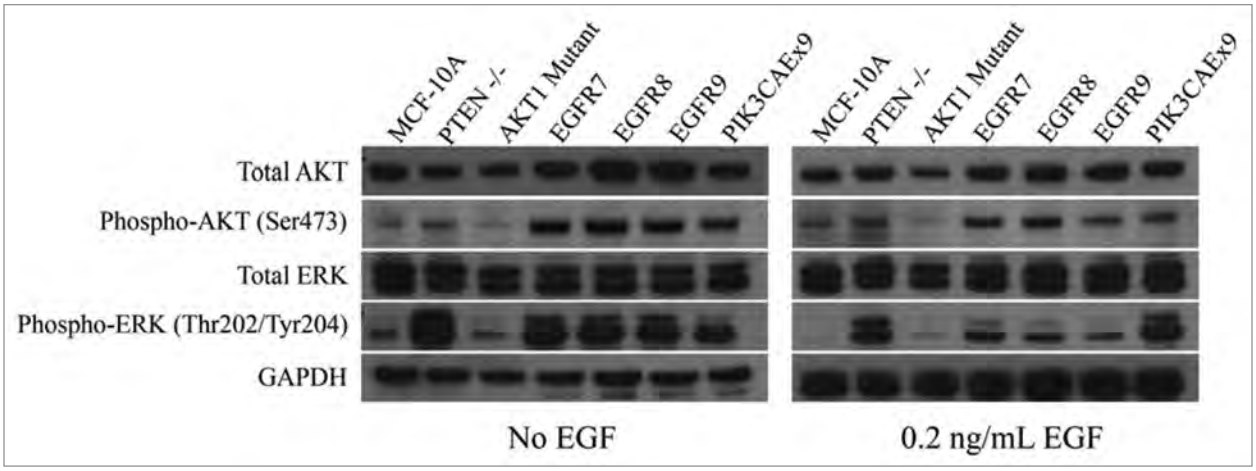


Figure 3. Alterations in the PI3K pathway activate multiple oncogenic pathways to varying degrees. Western blot demonstrates levels of total AKT, phosphorylated AKT(Ser473), total ERK and phosphorylated ERK (Thr202/Tyr204) in parental MCF-10A, *PTEN*$^{-/-}$, AKT1 mutant (E17K), EGFR7, EGFR8, EGFR9 (EGFR overexpressing clones) and *PIK3CAEx9* (E545K) cell lines in the absence of EGF (top panel) or presence of 0.2 ng/ml EGF (bottom panel). Cells were cultured and harvested at 24 hours after seeding as described in Materials and Methods. GAPDH is shown as a loading control. Results are representative of multiple independent experiments. (A and B) were independently obtained from each other. Direct comparisons across the panels cannot be made due to different exposure times of the western films.

to these cell lines in conditions with 0.2 ng/ml EGF (**Fig. 3** and right panel).

EGFR overexpressing clones, *PIK3CA* knock in cells and *PTEN*$^{-/-}$ cells also demonstrated activation of the MAPK pathway as displayed by the increased levels of phosphorylated Erk relative to total Erk both in the absence and presence of 0.2 ng/mL EGF (**Fig. 3**). Interestingly, in the absence of EGF the *PTEN*$^{-/-}$ cell lines exhibited a pronounced increase in Erk phosphorylation compared to the EGFR overexpressing cell lines or the *PIK3CA* knock in cell line (**Fig. 3** and left panel). However, in the presence of EGF, Erk phosphorylation in EGFR overexpressing clones was increased compared with parental and *AKT1* E17K cells, but was decreased relative to both *PTEN*$^{-/-}$ cells and *PIK3CA* knock in cells (**Fig. 3** and right panel). The cause for these differences are unknown, but these results are consistent with our previous observations in *PTEN*$^{-/-}$ cell lines showing that the presence or absence of EGF as well as duration of exposure to this growth factor can influence the level of Erk phosphorylation.[26] Thus, our biochemical analyses reaffirm that the presence of an *AKT1* E17K mutation alone does not confer significant oncogenic pathway signaling in human breast epithelial cells. In contrast, the presence of a *PIK3CA* oncogenic mutation, the loss of Pten, or overexpression of EGFR does indeed result in Akt and Erk phosphorylation in a manner similar to that found in breast cancer cells. However, the level and pattern of activation seen in these pathways is distinctly different between these three sets of cell lines, as evidenced by the varying levels of phosphorylation seen under conditions with and without exogenous EGF. This further underscores the previously unrecognized complexity of crosstalk that occurs between these important pathways.

EGFR overexpression sensitizes cells to lithium. Because the mechanism of action of lithium has not been fully elucidated, we wanted to assess whether EGFR overexpression, Pten loss or the *AKT1* E17K mutation could also predict for lithium sensitivity. There were two main reasons for formally testing this hypothesis. First, because one of lithium's targets has been suggested to be GSK3β, which is modulated by Akt and Erk activation, we hypothesized that EGFR overexpression and Pten loss via gene targeting would predict for lithium sensitivity based upon our previous data that *PTEN* knock out leads to increased Akt and Erk activation[26] and our own data presented in this study that EGFR overexpression also activates both the MAPK and PI3K pathways (**Fig. 3**). Second, despite the minimal pathway signaling seen with *AKT1* E17K knock in cells,[15] it was still formally possible that this mutation could activate other pathways that would lead to lithium sensitivity. There are in fact, examples where the same drug/compound can be extremely effective in various cancers with very different somatic alterations such as the case with *BCR/ABL* translocations in chronic myelogenous leukemia and certain *C-KIT* mutations in gastrointestinal stromal tumors both predicting for response to the small molecule inhibitor imatinib.[31] In addition, Wang et al. recently demonstrated that MLL leukemias are also sensitive to lithium treatment.[32]

We previously described the selective anti-neoplastic properties of lithium in vitro and in vivo using human breast and colon cancer cell lines that harbor activating mutations in *PIK3CA*.[25] A standard 10 mM concentration of lithium choloride (LiCl) was used in vitro based upon our initial tests[25] and the doses previously reported in studies examining the effects of lithium in various in vitro systems and their correlation to in vivo models.[32-36] It should be noted that although therapeutic lithium serum levels are 0.8–1.2 mEq/L, wide variations between serum lithium levels and intracellular concentrations of lithium have been reported.[37]

We hypothesized based on the biochemical results shown in **Figure 3**, that EGFR overexpression and *PTEN* loss, but not the *AKT1* E17K mutation would also predict for sensitivity to lithium. We therefore performed multiple growth assays with and without EGF to compare the growth inhibitory effects of lithium

in our panel of cell lines. As stated previously, the rationale for testing lithium toxicity under varying EGF conditions stems from our previous observations that differences in EGF concentration can have a profound effect on the downstream signaling cascades imparted by genetic mutations and their relative resistance and sensitivity to drugs such as rapamycin.[25] In addition, parental MCF-10A, *AKT1* E17K and empty vector control cells do not proliferate without EGF, but do proliferate with 0.2 ng/ml EGF and therefore can be used as isogenic counterparts when cultured with EGF. Importantly, we have previously described that parental MCF-10A cells and control cells proliferate at approximately an equal rate in 0.2 ng/ml EGF as mutant *PIK3CA* knock in cells in the absence of EGF, yet the former cell lines are resistant to lithium while the mutant *PIK3CA* clones were uniformly sensitive to lithium under these conditions. This strongly suggests that the effects of lithium are not simply due to increased cell proliferation.

Using identical conditions to our previous work, we found that treatment with LiCl significantly inhibited the growth of cells that overexpressed EGFR, similar to the response seen with the *PIK3CA* knock in cell line (**Fig. 4**). These effects were also observed at physiologic concentrations of EGF (**Fig. 4A vs. B**). Using a pair-wise comparison one-way ANOVA across cell lines, we found a statistically significant decrease in the proliferation of *PTEN*$^{-/-}$, EGFR#7, EGFR#8, EGFR#9 and PIK3CAEx9 as compared with parental MCF-10A cells ($p < 0.05$). In contrast, the parental MCF-10A and *AKT1* E17K cell lines were not significantly inhibited by LiCl when cultured in 0.2 ng/ml EGF ($p > 0.05$). As stated above, the effect of LiCl in parental MCF-10A and *AKT1* E17K cells could not be ascertained in the absence of EGF as these cells do not proliferate under these conditions. Interestingly, the *PTEN*$^{-/-}$ cell line demonstrated intermediate sensitivity to LiCl, when compared to the response seen with *PIK3CA* knock in and EGFR overexpressing cell lines. Thus, although the biochemical pathways activated by mutant *PIK3CA*, Pten loss and EGFR overexpression appear similar, they do not uniformly predict for sensitivity to lithium treatment.

To uncover the potential reasons for the differential responses to lithium seen in our panel of cell lines, we performed Western blotting to elucidate any biochemical changes in the MAPK or PI3K pathways elicited by lithium exposure. Although we have previously demonstrated an increase in total GSK3β in *PIK3CA* knock in cells upon lithium treatment,[25] this was not consistently observed in any of the EGFR overexpressing clones or PTEN$^{-/-}$ cells suggesting that increases in GSK3β are not the key mediator of lithium toxicity (data not shown). In addition, we also examined levels of phosphorylated p70S6Kinase, a marker of mTOR activation, but detectable levels were only present in *PIK3CA* mutant cells and EGFR overexpressing clones grown in 0.2 ng/ml EGF, with no appreciable change upon lithium exposure (**Sup. Fig. 2**). However, consistent differences in Akt and Erk phosphorylation were seen in lithium sensitive cell lines. For example, increased phosphorylation of Akt was seen at baseline in *PTEN*$^{-/-}$, EGFR7, EGFR8, EGFR9 and *PIK3CA* knock in cells and this was significantly reduced in the presence of LiCl in the EGFR overexpressing and *PIK3CA* knock in cells (**Fig. 5 and Sup. Fig. 3**). The decrease in Akt phosphorylation was far less pronounced in the *PTEN*$^{-/-}$ cells and was not appreciable in *AKT1* E17K cells consistent with the response to lithium treatment observed in the growth assays. In contrast, Erk phosphorylation was slightly decreased in *PTEN*$^{-/-}$ and *PIK3CA* knock in cells and moderately decreased in EGFR overexpressing clones upon lithium treatment when no EGF was added to the growth medium (**Fig. 5** and left panel and **Sup. Fig. 3**, left panel). However, marked decreases in Erk phosphorylation were seen in EGFR overexpressing cell lines and *PIK3CA* knock in cells when exposed to lithium under physiologic concentrations of EGF, but interestingly Erk phosphorylation appeared to be unaffected in *PTEN*$^{-/-}$ cell lines after lithium treatment under these conditions (**Fig. 5** and right panel). As expected, in all cases where lithium demonstrated some inhibition of proliferation, slight to moderate decreases in cyclin D1 protein were noted (**Fig. 5 and Sup. Fig. 3**).

The fact that lithium's effects were most pronounced on EGFR overexpressing and mutant *PIK3CA* knock in clones presented the intriguing possibility that perhaps lithium's effects were being mediated more proximally in the MAPK/PI3K pathways. To explore this hypothesis, we initially performed in vitro PI3K competitive kinase/ELISA assays in the presence and absence of lithium, but were unable to discern any consistent differences in PI3K in vitro activity (data not shown). We then performed Western blot analyses to examine EGFR tyrosine phosphorylation (1173), a marker of EGFR activation, in the presence and absence of lithium in EGFR overexpressing, mutant *PIK3CA* and parental MCF-10A control cells (**Sup. Fig. 4**). Interestingly, these results demonstrated that total EGFR levels decreased in parental MCF-10A, mutant *PIK3CA*, *PTEN* null and *AKT1* E17K cells in the presence and absence of EGF upon lithium exposure. Although there appears to also be decreased EGFR phosphorylation with lithium in EGF containing conditions, this is likely due to total EGFR levels decreasing. EGFR overexpressing clones however, did not show any appreciable change in total or phosphorylated EGFR with lithium treatment regardless of whether EGF was present in the media. However, due to the fact that decreases in total EGFR did not correlate with sensitivity to lithium, it can be concluded that changes in total EGFR protein or its phosphorylation are not responsible for the growth inhibitory effects of lithium in mutant *PIK3CA* and EGFR overexpressing cell lines.

Figure 4 (See opposite page). Lithium chloride inhibits the growth of EGFR overexpressing and *PIK3CAEx9* mutant cells but not MCF-10A parental or *AKT1* mutant cell lines. Cell proliferation and drug treatment assays were performed as described in Material and Methods with parental MCF-10A, *PTEN*$^{-/-}$, *AKT1* mutant (E17K), EGFR7, EGFR8, EGFR9 (EGFR overexpressing clones) and *PIK3CAEx9* (E545K) cell lines grown in (A) the absence of EGF or (B) presence of 0.2 ng/ml EGF. Note that MCF-10A and *AKT1* mutant cells could not be included in (A) since these cells do not proliferate in the absence of EGF. Bars represent the percentage of cell proliferation in 10 mM lithium chloride relative to cells grown in control medium (without lithium) after 6 days in culture. Error bars represent the standard error of the mean from triplicate samples. Results are representative of three independent experiments. *$p < 0.003$ compared to parental MCF-10A cells.

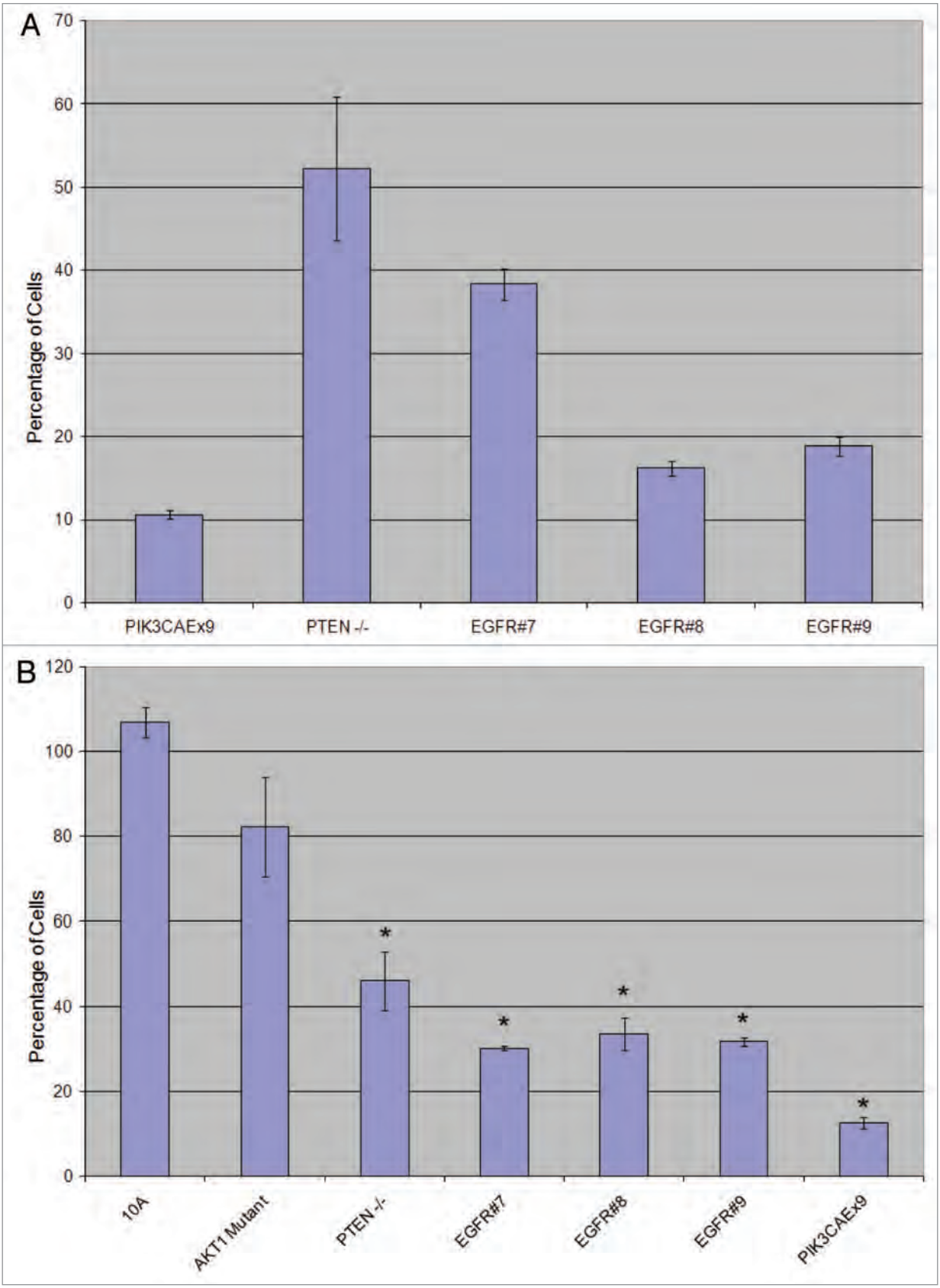


Figure 4. For figure legend, see page 362.

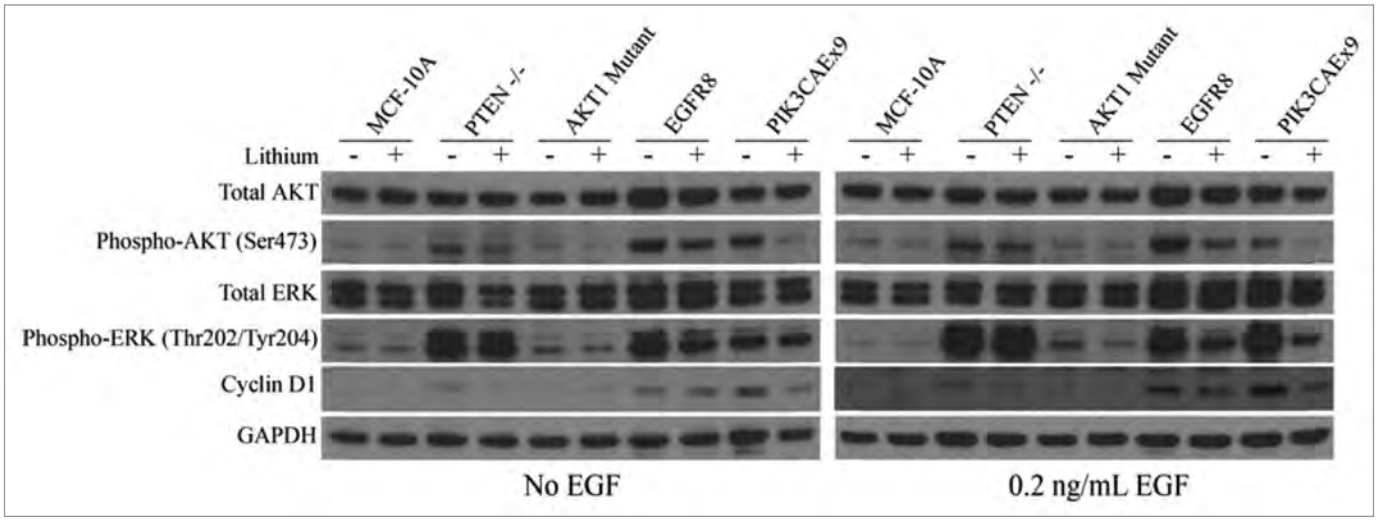


Figure 5. Mutant *PIK3CAEx9* and EGFR overexpressing clones demonstrate decreased Akt and Erk phosphorylation upon lithium treatment. Western blot demonstrating levels of total AKT, phosphorylated AKT (Ser473), total ERK and phosphorylated ERK (Thr202/Tyr204) in parental MCF-10A, *PTEN*$^{-/-}$, *AKT1* mutant (E17K), EGFR8, (representative EGFR overexpressing clone) and *PIK3CAEx9* (E545K) cell lines in the absence of EGF (left panel) or presence of 0.2 ng/ml EGF (right panel) in the absence (-) or presence (+) of 10 mM lithium chloride (lithium). Cells were cultured and harvested at 24 hours post drug treatment as described in Materials and Methods. GAPDH is shown as a loading control. Results are representative of multiple independent experiments. (Additional western blot analyses of EGFR overexpressing clones EGFR 7 and EGFR9 and empty vector control are shown in **Sup. Fig. 1**).

In sum, our results demonstrate that different genetic alterations within the same pathway are not biologically or functionally equivalent, as evidenced by the differences in biochemical pathway activation and responses to EGF stimulation. Moreover, because of the isogenic nature of our cell line panel, these data strongly suggest that genetic changes within a common pathway may not uniformly predict for sensitivity to a given pathway inhibitor.

Discussion

Cell-based models of human cancers have traditionally been used for drug discovery. Though their use has led to the development of effective therapies, lack of proper control cells has hindered the ability to validate a given compound's true target or mechanism of action and has often led to disappointing results in clinical trials. For example, the small molecule inhibitors gefitinib and erlotinib were originally proposed to be effective agents against lung cancer cells overexpressing EGFR although only 10% of patients responded to these drugs. Retrospective examination of these tumors demonstrated that certain EGFR mutations were associated with responses,[38-40] thus these mutations, rather than EGFR overexpression, have become better predictors of response to gefitinib and erlotinib.[41] Recently, the use of isogenic non-tumorigenic human breast epithelial cell lines has been proposed as a model for drug discovery using targeted gene replacement strategies.[24] Indeed, these studies demonstrated that EGFR mutations knocked into human breast epithelial cells dramatically sensitized cells to erlotinib thus recapitulating human clinical trial data. Additionally, prospective proof of principle concepts using isogenic cell lines to develop cancer therapies have been realized. A recent exciting clinical trial demonstrated the efficacy of poly (ADP-ribose) polymerase (PARP) inhibitors in BRCA mutation positive breast cancers.[42] This was the direct result of a "synthetic lethal" drug screen employing isogenic cell lines to identify PARP inhibitors' selectivity for BRCA null cells.[43,44] The data from these clinical trials demonstrate that better defined genetic models of cancer can ultimately result in highly effective targeted therapies and equally important, reliable predictors of response to those therapies.

Uncontrolled activity of the PI3K signaling pathway is found in many cancers. Our study further characterizes the disparate effects of various aberrations that occur within the PI3K pathway in human breast cancers using our unique library of isogenic cell lines. Loss of the tumor suppressor Pten is common in breast cancers.[8,9] We verify here that such loss is associated with increased constitutive activation of the MAPK and PI3K pathways; however, the level of Akt activation is not as extensive as that caused by a *PIK3CA* mutation and does not confer equivalent sensitivity to the toxic effects of lithium. In contrast to *PIK3CA* mutations and Pten loss, the presence of an isolated *AKT1* E17K mutation leads to minimal changes in the MAPK and PI3K pathways and as such, these cells appear relatively resistant to lithium compared to cells harboring an oncogenic *PIK3CA* mutation.

We demonstrate in this study that overexpression of EGFR leads to activation of multiple oncogenic pathways in a similar manner as somatic cell knock in of mutant *PIK3CA*. Thus a mutation of *PIK3CA* appears to mimic a proximal alteration resulting in the complex activation of multiple pathways including PI3K and MAPK signaling. Although activation of these two pathways by EGFR overexpression was expected based upon previous knowledge of RTK signaling, the signaling induced by mutant *PIK3CA* is confirmed to be of greater complexity as we previously reported in reference 25. We hypothesize that "rewiring" by mutant *PIK3CA* leads to MAPK pathway activation, perhaps

by virtue of the known binding that occurs via Ras and the Ras binding domain of p110α. Our results demonstrate that targeted therapies against key components in both the MAPK and PI3K pathways may yield the most effective results in cancers that harbor *PIK3CA* mutations. This may be especially relevant given the current inability to isolate mutant specific PI3K inhibitors. Interestingly, various inhibitors of the PI3K and MAPK pathways such as wortmannin, LY294002, U0126 and rapamycin have shown some activity in our *PIK3CA* knock in and *PTEN* knock out cell lines,[15,25,26] but depending on the culture conditions, non-selective toxicity due to off target effects was also present making interpretation of dual pathway inhibition difficult to assess.

Interestingly, loss of Pten led to only a partial sensitivity to lithium treatment despite baseline increases in Akt and Erk phosphorylation. However, in the absence of exogenous EGF, *PTEN* knock out cells had relatively less Akt phosphorylation and relatively more Erk phosphorylation compared to the highly lithium sensitive EGFR overexpressing and mutant *PIK3CA* cell lines. Intriguingly, lithium treatment of *PTEN*$^{-/-}$ cells modestly decreased Akt phosphorylation, yet there was no appreciable effect on Erk phosphorylation under physiologic EGF concentrations, a result that was distinct from EGFR overexpressing and mutant *PIK3CA* clones. Because of the isogenic nature of these cell lines, our results provide further evidence against the notion that pathway alterations in the PI3K pathway are functionally similar. During the preparation of this manuscript, Parsons and colleagues reported the newly discovered role of P-Rex2a in regulating Pten activity, as well as increased P-Rex2a activity in cancers with *PIK3CA* mutations.[45] It is tempting to speculate that changes in P-Rex2a activity may account for some of the differences seen in our isogenic system between *PTEN*$^{-/-}$ and mutant *PIK3CA* knock in cell lines.

Lithium, an FDA approved drug, is classically thought to be an inhibitor of GSK3β, but paradoxically, decreased tumor growth has been seen in many systems using lithium and other GSK3β inhibitors.[32-36] In our previous studies, we demonstrated that lithium decreases epithelial cell proliferation in cells harboring mutant *PIK3CA* and this was correlated with the upregulation of GSK3β, a known growth suppressor. However, in the current study, increases in GSK3β were not seen in EGFR overexpressing clones, despite the fact that these cell lines were otherwise phenotypically identical to mutant *PIK3CA* knock in cells (data not shown). More surprising was the finding that Akt and Erk, two molecular mediators of GSK3β phosphorylation and its subsequent inactivation,[35,46,47] demonstrated significantly decreased phosphorylation in EGFR overexpressing and mutant *PIK3CA* knock in cell lines upon lithium treatment. We conclude that significant activation of both PI3K and MAPK pathways beyond a critical threshold may be required for lithium sensitivity. These results suggest that lithium's mechanism of action may not be at the level of GSK3β, but rather more proximal in the signaling cascades induced by these oncogenic stimuli. We therefore hypothesized that lithium may affect more upstream events such as PI3K activity or phosphorylation of the EGFR itself. However, our analyses excluded these possibilities. The fact that Pten null cells displayed only partial sensitivity to lithium and did not display decreases in Erk phosphorylation with lithium treatment suggests once again that loss of Pten is functionally distinct from mutant *PIK3CA*. Equally important, because our experiments were performed in isogenic cell lines, direct and conclusive comparison of different genetic alterations within the same pathway can be made. Thus, our study suggests that the somatic changes that occur in human cancers may not be used interchangeably as predictors of response to a given pathway inhibitor as evidenced by our varying results with lithium sensitivity.

Our results have significant potential for clinical relevance beyond the ability to use isogenic cell lines to validate targets of therapy and predictive markers of response. Based on the experiments presented here, there is the possibility of using this information clinically for the treatment of breast cancer patients whose tumors harbor mutant *PIK3CA* and/or EGFR overexpression. For example, combination therapy with lithium may be a strategy to prevent drug resistance, since previous studies have noted that EGFR expression is associated with resistance to hormonal and chemotherapies in breast cancers.[48] In addition, the majority of breast tumors harboring a *PIK3CA* mutation are also hormone receptor positive, yet activation of the PI3K pathway is recognized as a mechanism of endocrine resistance,[49] and resistance to HER2-targeted agents.[50,51] Thus we envision that the addition of lithium therapy to current chemo, hormone and HER2 directed therapies may augment the clinical efficacy of these agents and perhaps reduce the emergence of drug resistant tumors.

Materials and Methods

Cell culture. MCF-10A, empty vector control and *AKT1* E17K mutant cells were maintained in DMEM:F12 supplemented with 5% horse serum, 20 ng/mL Epidermal Growth Factor (EGF), 10 μg/mL insulin, 0.5 μg/mL hydrocortisone and 0.1 μg/mL cholera toxin. All supplements were purchased from Sigma-Aldrich, St. Louis, MO. *PIK3CA* Exon 9 (E545K) knock in cells (PIK3CAEx9), *PTEN*$^{-/-}$ gene targeted clones of MCF-10A and EGFR overexpressing cells were grown in identical conditions except no EGF was added to the medium. EGFR overexpressing cells (clones labeled EGFR 7, EGFR 8, EGFR 9) were further supplemented with 180 μg/mL G418 (Invitrogen, Carlsbad, CA). All cell lines were grown at 37°C in 5% CO_2.

Stable transduction of epidermal growth factor receptor in human breast epithelial cells. A human EGFR cDNA was stably expressed in MCF-10A cells using the retroviral expression vector pFBneo, which was a kind gift from Dr. Anil K. Rustgi (University of Pennsylvania, PA). Retrovirus containing the coding sequence for EGFR was generated using Fugene6 (Roche Diagnostics, Indianapolis, IN) per the manufacturer's protocol in HEK-293T cells. Purified retrovirus was then used to infect MCF-10A cells following the manufacturer's protocol. Stable transformants were selected using 180 μg/mL G418 (Invitrogen, Carlsbad, CA). EGFR expression was confirmed by western blot using antibodies against total EGFR protein. Parental MCF-10A cells were also stably transduced in parallel with an empty retroviral expression vector pFBneo (named

Empty Vector or EV) and selected in the same manner to serve as controls for all experiments.

Cell proliferation assays. Cells were prepared by seeding each cell line in DMEM:F12 medium without phenol red, supplemented with 1% charcoal dextran-treated fetal bovine serum (Hyclone), 10 μg/mL insulin, 0.5 μg/mL hydrocortisone, 0.1 μg/mL cholera toxin at a density of 100,000 cells per 25 cm^2. Medium was changed to either EGF-free or 0.2 ng/mL EGF-containing medium in the absence and presence of 10 mM LiCl on days 1 and 4 as indicated. Cells were counted and evaluated for viability on days 1 and 6 using a Vi-CELL Cell Viability Analyzer (Beckman Coulter). All assays and growth conditions were performed in triplicate and repeated at least three times.

Immunoblotting. Lysates for cells grown in each experimental condition were prepared as previously described in reference 52. Western blotting was performed using the NuPage XCell SureLock electrophoresis system (Invitrogen, Carlsbad, CA) and PVDF membranes (Invitrogen, Carlsbad, CA). Primary antibodies were added overnight at 4°C, while secondary antibodies, conjugated with horseradish peroxidase were added for 1 hr at RT. Antibodies used in this study were anti-EGFR rabbit antibody (2232; Cell Signaling Technology), anti-phospho EGFR (Tyr 1173) rabbit anti-body (4407L; Cell Signaling Technology), anti-AKT rabbit antibody (9272; Cell Signaling Technology), anti-phospho AKT (Ser 473) rabbit antibody (9271; Cell Signaling Technology), anti-p42/p44 MAP kinase rabbit antibody (9102; Cell Signaling Technology), anti-phospho p42/p44 MAP kinase (Thr-202/Tyr-204) mouse antibody (9106; Cell Signaling Technology), anti-cyclin D1 rabbit antibody (2922; Cell Signaling Technology), anti-GSK3β rabbit antibody (9315; Cell Signaling Technology), anti-p70S6 Kinase rabbit antibody (9202; Cell Signaling Technology), anti-phospho p70S6 Kinase rabbit antibody (9205S; Cell Signaling Technology) anti-amphiregulin rabbit antibody (ab48191; Abcam), anti-TGFalpha mouse antibody (ab9578; Abcam) and anti-GAPDH mouse antibody (6C5) (ab8245; Abcam). All primary antibodies were used at 1:1,000 dilutions except the anti-GAPDH antibody, which was used at a 1:40,000 dilution. Blots were exposed to Kodak XAR film using chemiluminescence for detection (Perkin Elmer). All experiments were performed at least three times, with representative figures shown.

Transwell cell proliferation assays. Cells were seeded at approximately 10% confluency in bottom and top chambers of 6 well plates with transwell inserts (Cat# 3412, Corning) using DMEM:F12 medium without phenol red, supplemented with 1% charcoal dextran-treated fetal bovine serum (Hyclone), 10 μg/mL insulin, 0.5 μg/mL hydrocortisone, 0.1 μg/mL cholera toxin, without EGF. Medium was changed on days 1 and 4 and cells were then stained on day 6 with crystal violet to visualize cell proliferation. All experiments were performed in triplicate.

Statistical analyses. Standard error of the mean (SEM) was calculated for each proliferation assay. Statistical analyses were performed using a two-tailed Student's t-test and a one-way ANOVA across cell lines, which were calculated using Microsoft Excel and ezANOVA. A p value less than 0.05 was considered statistically significant.

Acknowledgements

We thank Anil Rustgi for providing the DNA vectors.

Financial Support

ASCO Young Investigator Award (M.J.H., D.P.C.); DOD Breast Cancer Research Program BC087658 (M.J.H.), W81XWH-06-1-0325 (J.P. Gustin), BC083057 (M.M.); KG090199 (J.D.L.), BCTR0707684 (B.H.P.); Flight Attendant Medical Research Institute (FAMRI) (J.D.L., H.K.), the V Foundation (J.D.L.), the Maryland Cigarette Restitution Fund (J.D.L.), the Avon Foundation (J.D.L., B.H.P.), NIH CA088843 (J.D.L., B.H.P.), CA109274 (J.P. Garay, B.H.P.), GM007309 (G.M.W.) CA009071 (D.P.C., D.J.); Susan G. Komen for the Cure PDF0707944 (A.M.A.) and the Breast Cancer Research Foundation (B.H.P.).

Disclosure of Potential Competing Interest

B.H.P. has received prior research funding from GlaxoSmithKline (GSK) though none of the studies reported here were supported by GSK. B.H.P. is a consultant for GSK and is on the scientific advisory board for Horizon Discovery LTD., and is entitled to payments for these services. These arrangements are managed according to the Johns Hopkins University conflict of interest policy.

Note

Supplemental materials can be found at:
http://www.landesbioscience.com/journals/cbt/article/14227

References

1. Cantley LC. The phosphoinositide 3-kinase pathway. Science 2002; 296:1655-7.
2. Volinia S, Hiles I, Ormondroyd E, Nizetic D, Antonacci R, Rocchi M, et al. Molecular cloning, cDNA sequence and chromosomal localization of the human phosphatidylinositol-3-kinase p110 alpha (PIK3CA) gene. Genomics 1994; 24:472-7.
3. Karakas B, Bachman KE, Park BH. Mutation of the PIK3CA oncogene in human cancers. Br J Cancer 2006; 94:455-9.
4. Samuels Y, Wang Z, Bardelli A, Silliman N, Ptak J, Szabo S, et al. High frequency of mutations of the PIK3CA gene in human cancers. Science 2004; 304:554.
5. Bachman KE, Argani P, Samuels Y, Silliman N, Ptak J, Szabo S, et al. The PIK3CA gene is mutated with high frequency in human breast cancers. Cancer Biol Ther 2004; 3:772-5.
6. Campbell IG, Russell SE, Choong DY, Montgomery KG, Ciavarella ML, Hooi CS, et al. Mutation of the PIK3CA gene in ovarian and breast cancer. Cancer Res 2004; 64:7678-81.
7. Saal LH, Holm K, Maurer M, Memeo L, Su T, Wang X, et al. PIK3CA mutations correlate with hormone receptors, node metastasis and ERBB2 and are mutually exclusive with PTEN loss in human breast carcinoma. Cancer Res 2005; 65:2554-9.
8. Depowski PL, Rosenthal SI, Ross JS. Loss of expression of the PTEN gene protein product is associated with poor outcome in breast cancer. Mod Pathol 2001; 14:672-6.
9. Perren A, Weng LP, Boag AH, Ziebold U, Thakore K, Dahia PL, et al. Immunohistochemical evidence of loss of PTEN expression in primary ductal adenocarcinomas of the breast. Am J Pathol 1999; 155:1253-60.
10. Saal LH, Johansson P, Holm K, Gruvberger-Saal SK, She QB, Maurer M, et al. Poor prognosis in carcinoma is associated with a gene expression signature of aberrant PTEN tumor suppressor pathway activity. Proc Natl Acad Sci USA 2007; 104:7564-9.
11. Carpten JD, Faber AL, Horn C, Donoho GP, Briggs SL, Robbins CM, et al. A transforming mutation in the pleckstrin homology domain of AKT1 in cancer. Nature 2007; 448:439-44.
12. Bleeker FE, Felicioni L, Buttitta F, Lamba S, Cardone L, Rodolfo M, et al. AKT1(E17K) in human solid tumours. Oncogene 2008; 27:5648-50.

13. Stemke-Hale K, Gonzalez-Angulo AM, Lluch A, Neve RM, Kuo WL, Davies M, et al. An integrative genomic and proteomic analysis of PIK3CA, PTEN and AKT mutations in breast cancer. Cancer Res 2008; 68:6084-91.
14. Kim MS, Jeong EG, Yoo NJ, Lee SH. Mutational analysis of oncogenic AKT E17K mutation in common solid cancers and acute leukaemias. Br J Cancer 2008; 98:1533-5.
15. Lauring J, Cosgrove DP, Fontana S, Gustin JP, Konishi H, Abukhdeir AM, et al. Knock in of the AKT1 E17K mutation in human breast epithelial cells does not recapitulate oncogenic PIK3CA mutations. Oncogene 2010; 29:2337-45.
16. Gupta S, Ramjaun AR, Haiko P, Wang Y, Warne PH, Nicke B, et al. Binding of ras to phosphoinositide 3-kinase p110alpha is required for ras-driven tumorigenesis in mice. Cell 2007; 129:957-68.
17. Gual P, Le Marchand-Brustel Y, Tanti JF. Positive and negative regulation of insulin signaling through IRS-1 phosphorylation. Biochimie 2005; 87:99-109.
18. Sharma AK, Horgan K, Douglas-Jones A, McClelland R, Gee J, Nicholson R. Dual immunocytochemical analysis of oestrogen and epidermal growth factor receptors in human breast cancer. Br J Cancer 1994; 69:1032-7.
19. Klijn JG, Look MP, Portengen H, Alexieva-Figusch J, van Putten WL, Foekens JA. The prognostic value of epidermal growth factor receptor (EGF-R) in primary breast cancer: results of a 10 year follow-up study. Breast Cancer Res Treat 1994; 29:73-83.
20. Harris AL, Nicholson S, Sainsbury JR, Farndon J, Wright C. Epidermal growth factor receptors in breast cancer: association with early relapse and death, poor response to hormones and interactions with neu. J Steroid Biochem 1989; 34:123-31.
21. Meche A, Cimpean AM, Raica M. Immunohistochemical expression and significance of epidermal growth factor receptor (EGFR) in breast cancer. Rom J Morphol Embryol 2009; 50:217-21.
22. Baselga J, Albanell J, Ruiz A, Lluch A, Gascon P, Guillem V, et al. Phase II and tumor pharmacodynamic study of gefitinib in patients with advanced breast cancer. J Clin Oncol 2005; 23:5323-33.
23. Corkery B, Crown J, Clynes M, O'Donovan N. Epidermal growth factor receptor as a potential therapeutic target in triple-negative breast cancer. Ann Oncol 2009; 20:862-7.
24. Di Nicolantonio F, Arena S, Gallicchio M, Zecchin D, Martini M, Flonta SE, et al. Replacement of normal with mutant alleles in the genome of normal human cells unveils mutation-specific drug responses. Proc Natl Acad Sci USA 2008; 105:20864-9.
25. Gustin JP, Karakas B, Weiss MB, Abukhdeir AM, Lauring J, Garay JP, et al. Knockin of mutant PIK3CA activates multiple oncogenic pathways. Proc Natl Acad Sci USA 2009; 106:2835-40.
26. Vitolo MI, Weiss MB, Szmacinski M, Tahir K, Waldman T, Park BH, et al. Deletion of PTEN promotes tumorigenic signaling, resistance to anoikis, and altered response to chemotherapeutic agents in human mammary epithelial cells. Cancer Res 2009; 69:8275-83.
27. Lauring J, Cosgrove DP, Fontana S, Gustin JP, Konishi H, Abukhdeir AM, et al. Knock in of the AKT1 E17K mutation in human breast epithelial cells does not recapitulate oncogenic PIK3CA mutations. Oncogene 2010; 29:2337-45.
28. Yoon DS, Wersto RP, Zhou W, Chrest FJ, Garrett ES, Kwon TK, et al. Variable levels of chromosomal instability and mitotic spindle checkpoint defects in breast cancer. Am J Pathol 2002; 161:391-7.
29. Soule HD, Maloney TM, Wolman SR, Peterson WD Jr, Brenz R, McGrath CM, et al. Isolation and characterization of a spontaneously immortalized human breast epithelial cell line, MCF-10. Cancer Res 1990; 50:6075-86.
30. Perez-Tenorio G, Alkhori L, Olsson B, Waltersson MA, Nordenskjold B, Rutqvist LE, et al. PIK3CA mutations and PTEN loss correlate with similar prognostic factors and are not mutually exclusive in breast cancer. Clin Cancer Res 2007; 13:3577-84.
31. Druker BJ. STI571 (Gleevec) as a paradigm for cancer therapy. Trends Mol Med 2002; 8:14-8.
32. Wang Z, Smith KS, Murphy M, Piloto O, Somervaille TC, Cleary ML. Glycogen synthase kinase 3 in MLL leukaemia maintenance and targeted therapy. Nature 2008; 455:1205-9.
33. Korur S, Huber RM, Sivasankaran B, Petrich M, Morin P Jr, Hemmings BA, et al. GSK3beta regulates differentiation and growth arrest in glioblastoma. PLoS One 2009; 4:7443.
34. Beurel E, Blivet-Van Eggelpoel MJ, Kornprobst M, Moritz S, Delelo R, Paye F, et al. Glycogen synthase kinase-3 inhibitors augment TRAIL-induced apoptotic death in human hepatoma cells. Biochem Pharmacol 2009; 77:54-65.
35. Kunnimalaiyaan M, Vaccaro AM, Ndiaye MA, Chen H. Inactivation of glycogen synthase kinase-3beta, a downstream target of the raf-1 pathway, is associated with growth suppression in medullary thyroid cancer cells. Mol Cancer Ther 2007; 6:1151-8.
36. Cao Q, Lu X, Feng YJ. Glycogen synthase kinase-3beta positively regulates the proliferation of human ovarian cancer cells. Cell Res 2006; 16:671-7.
37. El Balkhi S, Megarbane B, Poupon J, Baud FJ, Galliot-Guilley M. Lithium poisoning: is determination of the red blood cell lithium concentration useful? Clin Toxicol (Phila) 2009; 47:8-13.
38. Lynch TJ, Bell DW, Sordella R, Gurubhagavatula S, Okimoto RA, Brannigan BW, et al. Activating mutations in the epidermal growth factor receptor underlying responsiveness of non-small-cell lung cancer to gefitinib. N Engl J Med 2004; 350:2129-39.
39. Paez JG, Janne PA, Lee JC, Tracy S, Greulich H, Gabriel S, et al. EGFR mutations in lung cancer: correlation with clinical response to gefitinib therapy. Science 2004; 304:1497-500.
40. Pao W, Miller V, Zakowski M, Doherty J, Politi K, Sarkaria I, et al. EGF receptor gene mutations are common in lung cancers from "never smokers" and are associated with sensitivity of tumors to gefitinib and erlotinib. Proc Natl Acad Sci USA 2004; 101:13306-11.
41. Rosell R, Moran T, Queralt C, Porta R, Cardenal F, Camps C, et al. Screening for epidermal growth factor receptor mutations in lung cancer. N Engl J Med 2009; 361:958-67.
42. Fong PC, Boss DS, Yap TA, Tutt A, Wu P, Mergui-Roelvink M, et al. Inhibition of poly(ADP-ribose) polymerase in tumors from BRCA mutation carriers. N Engl J Med 2009; 361:123-34.
43. Farmer H, McCabe N, Lord CJ, Tutt AN, Johnson DA, Richardson TB, et al. Targeting the DNA repair defect in BRCA mutant cells as a therapeutic strategy. Nature 2005; 434:917-21.
44. Bryant HE, Schultz N, Thomas HD, Parker KM, Flower D, Lopez E, et al. Specific killing of BRCA2-deficient tumours with inhibitors of poly(ADP-ribose) polymerase. Nature 2005; 434:913-7.
45. Fine B, Hodakoski C, Koujak S, Su T, Saal LH, Maurer M, et al. Activation of the PI3K pathway in cancer through inhibition of PTEN by exchange factor P-REX2a. Science 2009; 325:1261-5.
46. Cross DA, Alessi DR, Cohen P, Andjelkovich M, Hemmings BA. Inhibition of glycogen synthase kinase-3 by insulin mediated by protein kinase B. Nature 1995; 378:785-9.
47. Ding Q, Xia W, Liu JC, Yang JY, Lee DF, Xia J, et al. Erk associates with and primes GSK-3beta for its inactivation resulting in upregulation of beta-catenin. Mol Cell 2005; 19:159-70.
48. Arpino G, Green SJ, Allred DC, Lew D, Martino S, Osborne CK, et al. HER-2 amplification, HER-1 expression and tamoxifen response in estrogen receptor-positive metastatic breast cancer: a southwest oncology group study. Clin Cancer Res 2004; 10:5670-6.
49. Campbell RA, Bhat-Nakshatri P, Patel NM, Constantinidou D, Ali S, Nakshatri H. Phosphatidylinositol 3-kinase/AKT-mediated activation of estrogen receptor alpha: a new model for anti-estrogen resistance. J Biol Chem 2001; 276:9817-24.
50. Nagata Y, Lan KH, Zhou X, Tan M, Esteva FJ, Sahin AA, et al. PTEN activation contributes to tumor inhibition by trastuzumab, and loss of PTEN predicts trastuzumab resistance in patients. Cancer Cell 2004; 6:117-27.
51. Berns K, Horlings HM, Hennessy BT, Madiredjo M, Hijmans EM, Beelen K, et al. A functional genetic approach identifies the PI3K pathway as a major determinant of trastuzumab resistance in breast cancer. Cancer Cell 2007; 12:395-402.
52. Konishi H, Karakas B, Abukhdeir AM, Lauring J, Gustin JP, Garay JP, et al. Knock-in of mutant K-ras in nontumorigenic human epithelial cells as a new model for studying K-ras mediated transformation. Cancer Res 2007; 67:8460-7.

Cancer Biology & Therapy 11:3, 368-370; February 1, 2011; © 2011 Landes Bioscience

Treating PIK3CA and EGFR overexpressing breast cancers with lithium citrate

Toni M. Brand and Deric L. Wheeler*

Department of Human Oncology; University of Wisconsin School of Medicine and Public Health; University of Wisconsin Comprehensive Cancer Center; Madison, WI USA

Key words: breast cancer, epidermal growth factor receptor (EGFR), lithium citrate, PIK3CA, receptor tyrosine kinase (RTK)

Abbreviations: EGFR, epidermal growth factor; GSK3, glycogen synthase kinase 3; PIP2, phosphatidylinositol-4,5-bisphosphate; PIP3, phosphoatidylinositol-3,4,5-triphosphate

Submitted: 12/29/10

Accepted: 12/30/10

DOI: 10.4161/cbt.11.3.14696

*Correspondence to: Deric L. Wheeler; Email: dlwheeler@wisc.edu

Commentary to: Higgins MJ, Beaver JA, Wong HY, Gustin JP, Lauring JD, Garay JP, et al. PIK3CA mutations and EGFR overexpression predict for lithium sensitivity in human breast epithelial cells. Cancer Biol Ther 2011; This issue.

The epidermal growth factor receptor (EGFR) is a membrane bound receptor tyrosine kinase of the ErbB/HER family. EGFR is most commonly expressed on cells of epithelial origin, however, it is found on the surface of most cells in the body. EGFR becomes activated at the cell surface by a variety of growth ligands, most notably the epidermal growth factor (EGF).[1] EGF binding to the extracellular domain of EGFR causes a conformational change that exposes EGFR's dimerization domain.[1] This conformational change allows EGFR to homo- or hetero-dimerize with other receptor tyrosine kinases, leading to activation of EGFR's intrinsic kinase activity and auto-phosphorylation of tyrosine residues on its C-terminal tail. These phosphorylation events allow for the association of various adaptor proteins, ultimately resulting in the activation of signaling pathways that result in cellular proliferation, migration and survival.

One of the major pathways activated by EGFR is the PI3K/AKT pathway.[2,3] PI3K, comprised of a p85 and p110 subunit, is recruited to the cell surface by binding to EGFR via its SH2 domain located on its p85 regulatory subunit. This event activates the p110 catalytic subunit of PI3K, which can phosphorylate phosphatidylinositol-4,5-bisphosphate (PIP2) to the second messenger phosphatidylinositol-3,4,5-triphosphate (PIP3) on the cellular membrane. Accumulation of PIP3 in the cell membrane leads to the recruitment of AKT via its pleckstrin homology (PH) domains. This recruitment results in the phosphorylation of AKT by phosphoinositide-dependent protein kinase 1 (PDK1) at T308 and at S473 by mTOR complex 2 (mTORC2) resulting in full activation of this protein kinase (reviewed in ref. 4). This reaction is reversed by the tumor suppressor protein phosphatase and tensin homolog (PTEN), further attenuating AKT signaling. PI3K can also be directly activated by the oncoprotein RAS. Activated AKT regulates a plethora of different cellular events, notably cellular proliferation and survival.

Genetic aberrations in several components of the PI3K pathway lead to dysregulated signaling in breast cancer. For example, somatic mutations have been reported in the gene encoding the p110α subunit of PI3K.[5] These mutations result in constitutively active PI3K independent of RAS or RTKs, leading to increased signaling through the AKT axis.[6,7] Additional mutations along this pathway include somatic mutations in AKT and PTEN, however, they are less frequent in breast cancers.[8] Most recently, the overexpression of EGFR has been identified in breast cancers and is associated with increased tumorigenic properties.[9] EGFR is estimated to be overexpressed in 65–72% of basal-like breast tumors and 50% of triple-negative breast tumors. Overall, the variety of aberrations that activate the PI3K/AKT signaling pathway in breast cancer is substantial and provides rationale for aggressively targeting this pathway for the treatment of this disease.

In this issue of *Cancer Biology & Therapy*, Higgins et al. investigate mutations in the PI3K signaling pathway to determine their predictive ability for sensitivity to lithium, an FDA approved

therapy for bipolar disorders. Specifically, the researchers created isogenic MCF-10A breast epithelial cell lines with various mutations along the PI3K/AKT pathway. The isogenic lines created include: (1) wild-type EGFR overexpressing cells, (2) PIK3CAEx9 E545K knock in cells (PIK3CA), (3) AKT1 E17K knock in cells (AKT1) and (4) PTEN knock out cells (PTEN$^{-/-}$). Their results showed that EGFR overexpression, *PIK3CA* mutation and PTEN$^{-/-}$ knock out cell lines have increased proliferation rates without EGF stimulation as compared to parental controls. Biochemical analysis of each isogenic cell line correlated this growth increase with activated AKT and MAPK protein levels. In the absence of EGF, EGFR overexpressing cells had increased AKT and MAPK activation, similar to PIK3CA. PTEN$^{-/-}$ cells, on the other hand, only demonstrated a minor increase in activated AKT, but a potent increase in pMAPK, while AKT1 mutants and parental cells demonstrated little to no activation of these proteins. EGF stimulation did not change the activation levels of AKT or MAPK in EGFR overexpressing cells, while PTEN$^{-/-}$ and PIK3CA cells demonstrated a potent increase in pMAPK. EGF stimulation did not affect the expression of pAKT or pMAPK in AKT1 mutants or parental cell lines. The differences revealed by these data support the findings that mutations along the PI3K/AKT axis are not functionally equivalent.

Based on these data, Higgins et al. concluded that aberrations along the same signaling pathway do not result in the same response to molecular targeting agents. In this publication they show that lithium citrate, a classic glycogen synthase kinase 3 (GSK3) inhibitor, can have selective anti-proliferative effects depending on the mutational status of various proteins along the PI3K/AKT pathway. In the isogenic model described, Higgins et al. demonstrate that EGFR overexpression and activating *PIK3CA* mutations are sensitive to lithium citrate. This decreased growth potential was observed under both EGF stimulated and non-stimulated conditions. Further, PTEN$^{-/-}$ cells demonstrated intermediate growth inhibition when stimulated with EGF, while the AKT1 mutants and parental cells remained insensitive. The anti-proliferative effects of lithium citrate were coupled to a decrease in pAKT and pMAPK with or without EGF stimulation in both EGFR overexpressing cells and PIK3CA mutants. Thus, different genetic alterations along the PI3K/AKT pathways result in differing responses to lithium citrate treatment.

Molecular predictors of response to molecular targeting agents have been intensely pursued in the last decade as a method to select and enrich patient populations that may respond to a given molecular targeting agent. When EGFR inhibitors were first tested in the clinic, it was postulated that EGFR expression level status of a cell could predict tumor cell response to these targeted therapies. However, intense clinical investigations have confirmed that EGFR expression does not serve as a reliable predictor of response to EGFR directed therapies.[10,11] Conversely, in 2004 it was reported that mutations of the *EGFR* in non-small-cell lung cancer (NSCLC) patients could predict response to EGFR inhibitors and represented a distinct, clinically relevant molecular subset of lung cancer. Molecular analysis of the EGFR in this patient population showed clinical responders harbored mutations in exon 19 characterized by in-frame deletions of amino-acids 747–750, exon 21 mutations resulting in L858R substitutions, and other mutations in exon 18 and 20. It was further determined that these mutations within the EGFR kinase domain lead to hyperactivity of the kinase and conferred a dependence on the mutated kinase for the survival of the NSCLC tumor cells. Further, this dependence on the EGFR signaling made tumor cells harboring these mutations exquisitely sensitive to EGFR inhibitors. The identification of these catalytic domain *EGFR* mutations that predict response to EGFR-TKIs in selected lung cancer patients represents a landmark development in the EGFR field and the field of biomarkers to targeted therapy (reviewed in ref. 12 and 13). Although these mutations served as biomarkers of response to EGFR TKI inhibitors, *KRAS* mutations have emerged as predictors of resistance to the EGFR antibody, cetuximab, in colorectal cancer (CRC). Lièvre et al. reported in 2006 that *KRAS* with mutations at codon 12 or 13 might be predictive of resistance to cetuximab therapy.[14] In this report, they analyzed 30 patients with metastatic CRC treated with cetuximab for *KRAS*, *BRAF* and *PIK3CA* mutations. *KRAS* mutations were found in 43% of tumors (13 tumors), and were significantly associated with resistance to cetuximab therapy ($p = 0.002$). To confirm these findings, Di Fiore et al. studied 59 patients with chemorefractory metastatic CRC treated with cetuximab plus chemotherapy. *KRAS* mutations were highly predictive of resistance to cetuximab plus chemotherapy.[15] Since these seminal findings, the predictive nature of *KRAS* mutations have borne out in several other clinical trials.[16-21] Together, these findings have strengthened our understanding of mutations of genes that predict response to targeted therapies. Thus, the study by Higgins et al. provides a very strong argument for identifying breast cancer populations that may respond to lithium.

To date, it is estimated that 1 in 8 women will be diagnosed with breast cancer in their lifetime (www.breastcancer.org).Modalities for treatment of breast cancer involve surgery, conventional chemotherapeutic regimens, radiation and now molecular targeting agents centered on the targeting of HER2 and the estrogen receptor. Lithium citrate, a drug traditionally used to treat biopolar disorder, has never been used for the treatment of breast cancer. However, this drug is approved by the FDA and is in wide use in the field of psychiatry. Although the introduction of a new therapeutic for the treatment of any type of human cancer is complex, the authors take a logical bottom up approach to identify patients where lithium based therapies may be beneficial (EGFR overexpressing and/or PI3KCA mutant breast cancers). Naturally, these results need to be validated with several breast cancer lines and primary breast tumors harboring these phenotypes. However, this approach of preclinical modeling of predictors of response to a particular chemotherapeutic/molecular targeting agent may save significant time for translating agents from the bench to the clinic.

References

1. Yarden Y, Sliwkowski MX. Untangling the ErbB signalling network. Nat Rev Mol Cell Biol 2001; 2:127-37.
2. Hynes NE, Lane HA. ERBB receptors and cancer: the complexity of targeted inhibitors. Nat Rev Cancer 2005; 5:341-54.
3. Sebolt-Leopold JS, Herrera R. Targeting the mitogen-activated protein kinase cascade to treat cancer. Nat Rev Cancer 2004; 4:937-47.
4. Engelman JA. Targeting PI3K signalling in cancer: opportunities, challenges and limitations. Nat Rev Cancer 2009; 9:550-62.
5. Miron A, Varadi M, Carrasco D, Li H, Luongo L, Kim HJ, et al. PIK3CA mutations in in situ and invasive breast carcinomas. Cancer Res 2010; 70:5674-8.
6. She QB, Chandarlapaty S, Ye Q, Lobo J, Haskell KM, Leander KR, et al. Breast tumor cells with PI3K mutation or HER2 amplification are selectively addicted to Akt signaling. Plos One 2008; 3:3065.
7. Zhao L, Vogt PK. Class I PI3K in oncogenic cellular transformation. Oncogene 2008; 27:5486-96.
8. Stemke-Hale K, Gonzalez-Angulo AM, Lluch A, Neve RM, Kuo WL, Davies M, et al. An integrative genomic and proteomic analysis of PIK3CA, PTEN and AKT mutations in breast cancer. Cancer Res 2008; 68:6084-91.
9. Burness ML, Grushko TA, Olopade OI. Epidermal growth factor receptor in triple-negative and basal-like breast cancer: promising clinical target or only a marker? Cancer J 16:23-32.
10. Mendelsohn J, Baselga J. The EGF receptor family as targets for cancer therapy. Oncogene 2000; 19:6550-65.
11. Baselga J. Does epidermal growth factor receptor status predict activity of cetuximab in colorectal cancer patients? Nat Clin Pract Gastr 2005; 2:284-5.
12. Pao W, Chmielecki J. Rational, biologically based treatment of EGFR-mutant non-small-cell lung cancer. Nat Rev Cancer 2010; 10:760-74.
13. Sharma SV, Bell DW, Settleman J, Haber DA. Epidermal growth factor receptor mutations in lung cancer. Nat Rev Cancer 2007; 7:169-81.
14. Lievre A, Bachet JB, Le Corre D, Boige V, Landi B, Emile JF, et al. KRAS mutation status is predictive of response to cetuximab therapy in colorectal cancer. Cancer Res 2006; 66:3992-5.
15. Di Fiore F, Blanchard F, Charbonnier F, Le Pessot F, Lamy A, Galais MP, et al. Clinical relevance of KRAS mutation detection in metastatic colorectal cancer treated by Cetuximab plus chemotherapy. Br J Cancer 2007; 96:1166-9.
16. De Roock W, Piessevaux H, De Schutter J, Janssens M, De Hertogh G, Personeni N, et al. KRAS wild-type state predicts survival and is associated to early radiological response in metastatic colorectal cancer treated with cetuximab. Ann Oncol 2008; 19:508-15.
17. Amado RG, Wolf M, Peeters M, Van Cutsem E, Siena S, Freeman DJ, et al. Wild-type KRAS is required for panitumumab efficacy in patients with metastatic colorectal cancer. J Clin Oncol 2008; 26:1626-34.
18. Karapetis CS, Khambata-Ford S, Jonker DJ, O'Callaghan CJ, Tu D, Tebbutt NC, et al. K-ras mutations and benefit from cetuximab in advanced colorectal cancer. N Engl J Med 2008; 359:1757-65.
19. Bokemeyer C, Bondarenko I, Hartmann J, De Braud F, Volovat C, Nippgen L, et al. KRAS status and efficacy of first-line treatment of patients with metastatic colorectal cancer (mCRC) with FOLFOX with or without cetuximab: The OPUS experience. ASCO Meeting Abstracts 2008; 4000.
20. Van Cutsem E, Lang I, D'haens G, Moiseyenko V, Zaluski J, Folprecht G, et al. KRAS status and efficacy in the first-line treatment of patients with metastatic colorectal cancer (mCRC) treated with FOLFIRI with or without cetuximab: the CRYSTAL experience. ASCO Meeting Abstracts 2008; 2.
21. Punt CJ, Tol J, Rodenburg CJ, Cats A, Creemers GM, Schrama JG, et al. Randomized phase III study of capecitabine, oxaliplatin and bevacizumab with or without cetuximab in advanced colorectal cancer (ACC), the CAIRO2 study of the Dutch Colorectal Cancer Group (DCCG). Ann Oncol. 2008; 19:734-8.

Clinical Cancer Research

Detection of Tumor *PIK3CA* Status in Metastatic Breast Cancer Using Peripheral Blood

Michaela J. Higgins, Danijela Jelovac, Evan Barnathan, et al.

Clin Cancer Res Published OnlineFirst March 15, 2012.

Published OnlineFirst on March 15, 2012; DOI:10.1158/1078-0432.CCR-11-2696

Clinical Cancer Research

Predictive Biomarkers and Personalized Medicine

See commentary by Richardson and Iglehart, p. 3209

Detection of Tumor *PIK3CA* Status in Metastatic Breast Cancer Using Peripheral Blood

Michaela J. Higgins[1], Danijela Jelovac[2], Evan Barnathan[2], Brian Blair[2], Shannon Slater[2], Penny Powers[2], Jane Zorzi[2], Stacie C. Jeter[2], George R. Oliver[3], John Fetting[2], Leisha Emens[2], Carol Riley[2], Vered Stearns[2], Frank Diehl[5], Philipp Angenendt[5], Peng Huang[2], Leslie Cope[2], Pedram Argani[2], Kathleen M. Murphy[4], Kurtis E. Bachman[6], Joel Greshock[6], Antonio C. Wolff[2], and Ben H. Park[2]

Abstract

Purpose: We sought to evaluate the feasibility of detecting *PIK3CA* mutations in circulating tumor DNA (ctDNA) from plasma of patients with metastatic breast cancer using a novel technique called BEAMing.

Experimental Design: In a retrospective analysis, 49 tumor and temporally matched plasma samples from patients with breast cancer were screened for *PIK3CA* mutations by BEAMing. We then prospectively screened the ctDNA of 60 patients with metastatic breast cancer for *PIK3CA* mutations by BEAMing and compared the findings with results obtained by screening corresponding archival tumor tissue DNA using both sequencing and BEAMing.

Results: The overall frequency of *PIK3CA* mutations by BEAMing was similar in both patient cohorts (29% and 28.3%, respectively). In the retrospective cohort, the concordance of *PIK3CA* mutation status by BEAMing between formalin-fixed, paraffin-embedded (FFPE) samples and ctDNA from temporally matched plasma was 100% (34 of 34). In the prospective cohort, the concordance rate among 51 evaluable cases was 72.5% between BEAMing of ctDNA and sequencing of archival tumor tissue DNA. When the same archival tissue DNA was screened by both sequencing and BEAMing for *PIK3CA* mutations ($n = 41$ tissue samples), there was 100% concordance in the obtained results.

Conclusions: Analysis of plasma-derived ctDNA for the detection of *PIK3CA* mutations in patients with metastatic breast cancer is feasible. Our results suggest that *PIK3CA* mutational status can change upon disease recurrence, emphasizing the importance of reassessing *PIK3CA* status on contemporary (not archival) biospecimens. These results have implications for the development of predictive biomarkers of response to targeted therapies. *Clin Cancer Res; 18(12); 1–8. ©2012 AACR.*

Introduction

Aberrant phosphoinositide 3-kinase (PI3K) pathway signaling is being studied as a prognostic marker in breast cancer and as a predictive marker for targeted-specific therapies (1–5). The gene encoding the p110α catalytic domain of PI3K, *PIK3CA*, is the most commonly mutated oncogene in breast cancer, and more than 80% of somatic *PIK3CA* mutations occur in one of 3 recurrent "hotspot" locations (6–8). Key to the interpretation and success of clinical trials targeting the PI3K pathway is the accurate identification of tumors with sensitizing or desensitizing mutations to specific drugs, as observed with *EGFR* mutations in non–small cell lung cancer, somatic *KRAS* mutations in colorectal cancers, and *BRAF* mutations in metastatic melanoma (9–13). Determination of somatic mutational status traditionally requires freshly obtained or archival biopsy specimens for sequencing. However, the contamination of tumor samples with normal tissue, tumor heterogeneity, and variable quality of extracted and stored DNA can interfere with accurate analyses (14). Therefore, current methods routinely used to detect mutations in DNA from formalin-fixed, paraffin-embedded (FFPE) specimens are limited, and more accurate and less invasive detection methods are needed. The ability to detect tumor DNA mutations in a blood sample (i.e., a liquid biopsy) would allow an easy to obtain,

Authors' Affiliations: [1]Massachusetts General Hospital Cancer Center, Boston, Massachusetts; [2]Sidney Kimmel Comprehensive Cancer Center at Johns Hopkins, Baltimore, Maryland; [3]Center for Clinical Innovation, Parkland Health and Hospital System; [4]ProPath, Dallas, Texas [5]Inostics, GmbH, Hamburg, Germany; and [6]GlaxoSmithKline, Oncology R&D, Collegeville, Pennsylvania

Note: Supplementary data for this article are available at Clinical Cancer Research Online (http://clincancerres.aacrjournals.org/).

The results of this study were presented in part at the American Society of Clinical Oncology Annual Meetings, Chicago, IL, June 2010 and 2011.

Corresponding Authors: Ben H. Park, The Sidney Kimmel Comprehensive Cancer Center at Johns Hopkins, 1650 Orleans Street, Rm 151, Baltimore, MD 21287. Phone: 410-502-7399; Fax: 410-614-4073; E-mail: bpark2@jhmi.edu; and Antonio C. Wolff. Phone: 410-614-4192; Fax: 410-614-4073; E-mail: awolff@jhmi.edu

doi: 10.1158/1078-0432.CCR-11-2696

Published OnlineFirst on March 15, 2012; DOI:10.1158/1078-0432.CCR-11-2696

Translational Relevance

We report a prospective study on the feasibility and accuracy of screening for the presence of tumor *PIK3CA* mutations in patients with metastatic breast cancer using blood as a tissue source by way of a novel and robust assay called BEAMing. We observed significant discordance between testing of primary early stage tumors compared with testing at a later time point after cancer recurrence. We describe that loss or gain of *PIK3CA* mutations can occur with metastatic disease in up to 20% of cases. Because multiple inhibitors of phosphoinositide 3-kinases are currently in development, an accurate determination of current *PIK3CA* mutation status has clear implications for trial design and future clinical practice. Our results suggest that BEAMing provides an accurate and relatively noninvasive method for current ascertainment of *PIK3CA* mutational status in patients with metastatic breast cancer.

noninvasive, and quantifiable method for use in the clinical setting to identify candidates for specific therapies and monitoring of disease status over time. It would also provide real-time assessment of mutational status without having to rely on archival specimens from the original primary tumor (if available) or the need for invasive biopsy procedures of a metastatic site.

DNA containing somatic mutations is highly tumor specific and thus can potentially be used as a biomarker. A novel technique for identifying cell free, plasma-derived circulating mutant DNA termed "BEAMing" has recently been developed. BEAMing is named after the 4 key components of the method (Beads, Emulsification, Amplification, and Magnetics; refs. 15, 16). Assessment of circulating tumor DNA (ctDNA) by BEAMing can provide the mutational status of a patient's cancer (17, 18). BEAMing can be carried out on virtually any tissue source without enriching for tumor cells, and thus the risk of "masking" mutations due to tumor heterogeneity and/or contamination of normal cells is greatly reduced. Use of plasma (peripheral blood) offers many advantages such as ease of access and the ability to repeat tests over time as the source of DNA is continuous.

We designed this study to test the feasibility of using BEAMing on ctDNA to determine *PIK3CA* mutational status in peripheral blood of patients with breast cancer. An initial retrospective cohort analysis used 49 paired patient samples of tumor tissue and blood obtained at the same time. We then conducted a separate confirmatory, prospective study to determine the feasibility of using BEAMing to detect *PIK3CA* mutations in patients with recurrent metastatic breast cancer (blood and tissue) and compared it to standard DNA sequencing methods currently used. We describe rates of observed mutation detection and concordance, and the resulting critical implications for clinical practice and research studies.

Materials and Methods

Retrospective contemporaneous tissue and blood collection cohort

Paired samples of breast cancer tumor tissue and blood samples taken from the same patients on the same day were obtained by Indivumed GmbH as part of their tissue repository service. In 45 of these cases, the tumor specimen collected was the primary breast tumor and a biopsy of a metastatic breast cancer lesion was the tissue obtained for the remaining 4. The collection of biospecimens and clinical data within the Federal republic of Germany is not regulated by national law; these samples were collected ethically within the framework of the "Hamburger Krankenhausgesetz 12a". Genomic DNA was extracted from 49 tissue samples and BEAMing used to identify one of 3 mutations in the *PIK3CA* gene: (Ex 9 1633G>A E545K; Ex 20 3140A>G H1047R; Ex 20 3140A>T H1047L). ctDNA derived from plasma from all patients in whom a *PIK3CA* mutation was identified in the tissue samples, and also from 20 randomly selected patients whose tumors were *PIK3CA* wild-type, were subsequently analyzed by BEAMing.

Prospective feasibility study patient cohort

Women with metastatic breast cancer were prospectively enrolled at The Johns Hopkins Sidney Kimmel Comprehensive Cancer Center (Baltimore, MD). Patient characteristics are shown in Supplementary Table S1. All subjects were required to have a prior breast cancer tissue sample available and be willing to provide a 10 mL peripheral venous blood sample. Review of medical records was conducted to confirm sites of metastatic disease and histopathologic features of the primary breast tumor. The protocol was approved by the Johns Hopkins Institutional Review Board and informed consent was obtained from all patients. Of note, blood for ctDNA was obtained at the time of trial entry, and then archival tumor tissue was retrieved from pathologists, however these tissue specimens had been acquired months to years before study entry.

Tissue sequencing

A hematoxylin and eosin–stained slide of each patient's tumor (primary or metastatic) was used for identification of tumor by the study pathologist. Areas of tumor tissue were removed from serial unstained slides (10μ) using the Zymo pen and Pinpoint solution (Zymo Research) as per the manufacturer's protocol. The percentage of tumor cells in the resultant samples was approximately 80% to 90%. DNA was purified using QIAamp DNA spin columns (Qiagen). *PIK3CA* exons 9 and 20 were PCR amplified using one biotinylated primer in each reaction. Following amplification, the biotinylated PCR products were purified and sequenced using the Pyromark Q24 (Qiagen) with sequencing primers designed to identify the following mutations: *PIK3CA* Ex 9 1624G>A E542K; *PIK3CA* Ex 9 1633G>A

Published OnlineFirst on March 15, 2012; DOI:10.1158/1078-0432.CCR-11-2696

E545K; and *PIK3CA* Ex 20 3140A>G H1047R. Two known *PIK3CA* mutation-positive cell lines, HCT-15 (E545K) and HCT-116 (H1047R), were run as controls (19). Investigators conducting the sequencing assays were blinded to the BEAMing results.

BEAMing

Plasma was derived from blood samples by centrifugation within 2 hours of collection and were spun twice for each sample to ensure the absence of cellular contamination. Free circulating DNA was isolated from plasma samples by the QIAamp DNA purification kit (Qiagen). After sequencing had been conducted on the genomic DNA from the archival tissue samples, the remaining DNA if available, was also used for BEAMing. ctDNA isolated from plasma and BEAMing assays were conducted on each sample by Inostics GmbH. BEAMing is a technique in which individual DNA molecules are attached to magnetic beads in water-in-oil emulsions and then subjected to compartmentalized PCR amplification. The mutational status of DNA bound to beads is then determined by hybridization to fluorescent allele-specific probes for mutant or wild-type *PIK3CA*. Flow cytometry is then used to quantify the level of mutant DNA present in the plasma (15, 17). For the retrospective contemporaneous tissue and blood collection cohort analysis, Ex 9 1633G>A E545K; Ex 20 3140A>G H1047R; Ex 20 3140A>T H1047L mutations were queried by BEAMing (at the time of the retrospective study, BEAMing for the Ex 9 E542K mutation was not available). For the prospective study, *PIK3CA* Ex 9 1624G>A E542K; *PIK3CA* Ex 9 1633G>A E545K; and *PIK3CA* Ex 20 3140A>G H1047R were analyzed by BEAMing. To contain costs, the Ex 20 3140A>T H1047L was not conducted in the prospective study. Investigators conducting the BEAMing assays were blinded to the sequencing results.

Statistical analysis

In the statistical analyses, each BEAMing test result was treated as an independent variable under the assumption that inaccuracies in detecting a single base pair substitution with either method would not influence the test result using the other method. The mechanical ability of BEAMing to detect specific mutations in tumor tissue identified by standard sequencing methods was tested by directly comparing results obtained by the 2 assay methods in the tumors from the prospective patient cohort (same tumor, different assay). The ability of BEAMing to identify the same specific mutations previously detected by standard sequencing of tumor tissue in circulating DNA was done by using the blood that was collected at the time of surgery (i.e., the retrospective patient cohort). Finally, the hypothesis that the mutational status of patients with breast cancer would not change over time was tested by comparing the ctDNA by BEAMing to surgical specimens that had been previously collected (i.e., the prospective study cohort). Where appropriate, the concordance between platforms and tissues was tested with Kappa statistics, which is a conservative measurement of relative agreement between 2 categorical items (in this case mutant vs. wild-type) that takes into account agreement by chance. Kappa ranges from $\kappa = 1$ (perfect agreement) to $\kappa = 0$ (no agreement other than would be expected by chance). Calculations of chance overlap with confidence intervals (CI) were used to estimate significance of each calculation based on the observed data. Expected concordance rates were calculated using a collation of the mutation frequencies of *PIK3CA* detected in previous studies of breast cancers (19). On the basis of these data, the expected frequencies of mutations in the prospective cohort were estimated at 13.3%, 4.4%, and 2.5% for variants 3140A>G, 1633G>A, and 1624G>A, respectively (collectively 20.1%), whereas in the retrospective cohort study the expected frequencies were estimated at 13.3%, 4.4%, and 1.1% for mutations 3140A>G, 1633G>A, and 3140A>T, respectively (collectively 18.7%). Because of the anticipated large fraction of 'wild-type' scores, expected concordance rates were tested against the hypothesis that either assay would detect mutations at the expected frequency.

Results

Patients

Between January 2004 and 2009, samples from 49 patients with metastatic breast cancer (median age, 62; range, 39–84) were collected in the retrospective cohort from Germany. Thirty-five (71.4%) patients had estrogen receptor–positive disease. HER2 status was unavailable for some patients as shown in Table 1. In 45 of 49 cases, tumor tissue was collected from the primary breast tumor on the same day as the blood sample for BEAMing. Four tissue samples came from a metastatic breast tumor site with blood samples also collected on the same day of biopsy.

Between February 2010 and May 2010, 60 patients (median age, 56; range, 36–85) were enrolled in the prospective feasibility study at Johns Hopkins. All had histologically confirmed breast cancer with radiological and/or pathologic evidence of distant recurrent metastatic disease. Most patients (65%) had estrogen receptor–positive breast cancer, 6 (10%) had triple-negative disease, and 20 (33.3%) tumors showed overexpression of HER2. The source of archival tissue sample was primary tumor for 42 (80.8%) participants and biopsy of a distant metastatic tumor site for 10 (19.2%).

Retrospective cohort

For the retrospective cohort, where blood was collected at the time of tumor biopsy, the goal was to determine whether BEAMing could reliably detect mutations in ctDNA that were identified by direct BEAMing analysis of tumor tissue. Mutations were identified by BEAMing of FFPE-derived genomic DNA in 14 of 49 patients (29%). BEAMing of plasma ctDNA from the same patients identified the same 14 mutations as observed by BEAMing the FFPE-derived tissue samples (Table 1). BEAMing of 20 FFPE-derived genomic DNA wild-type *PIK3CA* samples was 100%

Published OnlineFirst on March 15, 2012; DOI:10.1158/1078-0432.CCR-11-2696

Table 1. Spectrum of *PIK3CA* mutations identified retrospectively in peripheral blood of patients with breast cancer by BEAMing of ctDNA and also by BEAMing of breast tumor tissue collected simultaneously from the same patients

Mutation	Amino acid change	Observed frequency in both ctDNA and tumor tissue/expected frequency[a] N = 49 (%)/(%)	Tumor hormone receptor positive N (%)	HER2 status of tumor N (%)	"Triple-negative" tumor
1633G>A	545E>K	3 (6.1)/(4.4)	3 (100)	2 (75) negative 1 (25) unknown	0
3140A>G	1047H>R	10 (20.4)/(13.3)	7 (70)	4 (40) positive 5 (50) negative 1 (10) unknown	1
3140A>T	1047H>L	1 (2)/(1.1)	1 (100)	1 (100) positive	0
Total number of mutations		14 (28.6)/(18.7)	11 (78.6)	5 (35.7) positive 7 (50) negative 2 (14.3) unknown	1 (7.1)

[a]Distribution of somatic mutations in PIK3CA, Catalogue Of Somatic Mutations in Cancer (COSMIC)

concordant with BEAMing of corresponding ctDNA. These results suggest that ctDNA BEAMing accurately detects the *PIK3CA* mutational status in patients with breast cancer when testing a blood sample collected at the time of tissue biopsy ($\kappa = 1.0$). The actual agreement did not overlap with what would be expected by chance for the retrospective study ($\kappa = 0.070$; 95% CI, 0.0–0.57).

Prospective cohort

In a prospective setting, a 10-mL blood sample was obtained from patients with recurrent metastatic breast cancer ($n = 60$) at the time of study entry. For each patient, consent to retrieve archival tumor tissue for DNA analysis was also obtained. The median time between blood draw at study entry and time that the tumor specimen was removed from the patient was 5.0 years (range, 0.26–23.9 years). ctDNA was successfully extracted from all patients. Archival tumor tissue with adequate DNA for standard sequencing was obtained in 51 cases, whereas tumor from 9 patient cases had insufficient tissue or no archival tissue available.

Oncogenic mutations were detected in 28.3% of plasma samples (Table 2) and most were in exon 20. *PIK3CA*

Table 2. Distribution of *PIK3CA* mutations detected prospectively in plasma of patients with metastatic breast cancer by BEAMing and correlation with hormone receptor and HER2 status of primary tumor

Mutation	Amino acid change	Observed frequency/ expected frequency[a] N (%)/(%)	Primary tumor hormone receptor positive N (%)	Primary tumor HER2 status N (%)	Primary tumor "triple negative"
1624G>A	542E>K	3 (5)/(2.5)	2 (66.6)	1 (33.3) positive 2 (66.6) negative	1 (33.3)
1633G>A	545E>K	4 (6.7)/(4.4)	3 (75)	3 (75) positive 1 (25) negative	0
3140A>G	1047H>R	12 (20)/(13.3)	11 (91.7) 1 (8.3)	4 (33.3) positive 8 (66.6) negative	0
Total number of mutations[b]		17 (28.3)/(20.1)	Among 17 samples with at least 1 mutation detected: 14 (82.4)	Among 17 samples with at least 1 mutation detected: 7 (41.2) positive 10 (58.8) negative	1 (5.9)

[a]Distribution of somatic mutations in PIK3CA, Catalogue Of Somatic Mutations in Cancer (COSMIC).
[b]Nineteen mutations were identified in 17 patient samples.

Published OnlineFirst on March 15, 2012; DOI:10.1158/1078-0432.CCR-11-2696

Table 3. Results of standard sequencing of archival tissue tumor specimens and mutations detected by BEAMing of ctDNA in prospective study

Description of tissue tumor DNA and plasma-derived ctDNA for mutation analysis	Number of samples (%)
Number of samples with adequate archival tissue available for sequencing	51/60 (85)
Number of samples with adequate plasma available for ctDNA extraction and BEAMing	60/60 (100)
Number of *PIK3CA* mutations identified by sequencing archival tissue	14/51 (27.4)
Number of *PIK3CA* mutations identified by BEAMing of plasma-derived ctDNA[a]	17/60 (28.3)
Number of samples that were *PIK3CA* wild-type by sequencing of archival tissue and *PIK3CA* wild-type by BEAMing of plasma-derived ctDNA	37/51 (72.5)
Number of samples that were *PIK3CA* mutant by sequencing of archival tissue and *PIK3CA* mutant by BEAMing of plasma-derived ctDNA	8/51 (15.6)
Number of samples that were *PIK3CA* wild-type on sequencing of archival tissue and *PIK3CA* mutant by BEAMing of plasma-derived ctDNA	8/51 (15.6)
Number of samples that were *PIK3CA* mutant on sequencing of archival tissue and *PIK3CA* wild-type by BEAMing of plasma-derived ctDNA	6/51 (11.7)

NOTE: N = 60 samples available for ctDNA extraction and BEAMing. N = 51 samples available for sequencing.
[a]Nineteen mutations were identified by BEAMing in 17 patient samples.

mutations were most commonly observed in patients with hormone receptor–positive breast cancer and in HER2-positive breast cancers. Of interest, BEAMing of plasma samples from 2 patients showed 2 separate *PIK3CA* mutations in each of exons 9 and 20.

PIK3CA mutations were detected by sequencing of archival tissue in 14 samples (27.5%, see Table 3). While the ctDNA mutation profile in the retrospective cohort (tissue and blood collected at the same time) mimicked that of the tumor tissues, discordant results (pos/neg and neg/pos) were seen in 14 of 51 samples (blood sample compared with archival tumor). Six patients had mutant *PIK3CA* in archival tissues with no mutation detected in blood whereas 8 other patients had the opposite profile with normal archival tissue but mutated *PIK3CA* in blood (κ = 0.4829; CI, 0.22–074). Although the agreement is better than what would have been expected by chance (κ = 0.0366; CI, 0.0–0.45), the agreement fell significantly below the real-time estimates of *PIK3CA* mutations from ctDNA when blood was collected at the time of tissue biopsy (where $\kappa = 1.0$).

In view of the observed tissue/blood discordance observed in the prospective cohort, we decided to also test these tissues by BEAMing as was done in blood. Of the 51 prospective tissue samples initially tested by standard sequencing, enough DNA remained in 41 of them for BEAMing allowing cross-platform comparisons to assess *PIK3CA* status (same tissue, different assay). As shown in Table 4, there was 100% concordance between BEAMing and standard sequencing when assessing *PIK3CA* mutational status in these 41 tissue samples ($\kappa = 1.0$). The calculated $\kappa = 0.036$ (95% CI, 0.0–0.53) for chance agreement of this direct comparison of platforms.

Discussion

We report the feasibility of screening for the presence of common oncogenic *PIK3CA* mutations in patients with breast cancer by a simple blood test using BEAMing. Overall, ctDNA was isolated from 109 patient blood samples and a *PIK3CA* mutation was identified in 28.4%. Of critical importance, testing done in the prospective cohort clearly exemplified the current challenges of locating quality archival tissue samples for biomarker testing. In this case, sufficient tissue was available for only 51 of 60 prospectively enrolled patients (85%). In contrast, BEAMing on ctDNA

Table 4. Concordance of *PIK3CA* mutational status detected by sequencing or by BEAMing of DNA derived from the same tissue specimen (N = 41 matched samples, %)

Number of samples with adequate archival tissue available for BEAMing	41 (68.3)
Number of samples with adequate plasma available for ctDNA extraction and BEAMing	60 (100)
Number of samples that were *PIK3CA* wild-type by sequencing of archival tissue and *PIK3CA* wild-type by BEAMing of archival tissue	30 (73.1)
Number of samples that contained the same *PIK3CA* mutation by sequencing of archival tissue and by BEAMing of archival tissue	11 (26.8)
Number of discordant results between sequencing of archival tissue and BEAMing of archival tissue	0

Published OnlineFirst on March 15, 2012; DOI:10.1158/1078-0432.CCR-11-2696

was successful in the blood samples from all 60 enrolled patients, with a *PIK3CA* mutation frequency similar to that previously reported (Table 2; refs. 6, 7, 20, 21). Of interest, BEAMing of plasma samples from 2 patients showed 2 separate *PIK3CA* mutations in each of exons 9 and 20; this is a rare phenomenon but has been previously described (22).

Most critical and with implications for both clinical trial design and clinical practice, we observed a significant discordance between mutation analysis using current versus archival tissue. In the retrospective cohort, the concordance between *PIK3CA* mutational status by BEAMing in blood and by standard sequencing in tissue was 100%. However, a 27.5% discordance was observed among 51 patients with recurrent metastatic disease prospectively tested by BEAMing in blood compared with standard sequencing of archival tissue obtained months to years before study entry. There are several important implications of this observation. It is theoretically possible that BEAMing failed to detect a *PIK3CA* mutation and provided a "false-negative" result in the cases that were mutation positive by sequencing of tissue yet wild-type by BEAMing of ctDNA. We feel this is unlikely given the previously reported, extremely high sensitivity of the BEAMing technique (23), and the fact that all *PIK3CA* mutations in tissue samples were detected in ctDNA by BEAMing in the retrospective study. That said, we cannot formally exclude this as a possibility with our current samples and data. As a potential explanation, it is possible that some patients had very little disease burden at the time of blood draw, therefore, plausibly BEAMing may not have been sensitive enough to identify *PIK3CA* mutations in this setting. However, reexamination of our patients' recurrent disease status did not show this in cases where discordant results occurred. Notably, we did observe that discordant results were only seen in patients whose archival tumor specimen was at least 3 years before blood draw for ctDNA (Supplementary Table S2). An ongoing study will establish the lower threshold of *PIK3CA* mutation detection using early, operable patients with breast cancer who will have BEAMing conducted on ctDNA both before and after surgical resection of their tumors.

Currently, many trials testing PI3K inhibitors are enrolling only patients with mutant *PIK3CA* tumors. Our findings suggest that in patients whose archival tumor specimen was obtained 3 or more years before blood draw for ctDNA, *PIK3CA* mutational status may change, as paired samples from 8 patients (15.6%) showed wild-type *PIK3CA* in their archival tissue sample and mutant *PIK3CA* in their peripheral blood when recurrent metastatic disease was present, whereas paired samples from 6 other patients (11.7%) were discordant in the opposite direction (mutant to wild-type). Our findings support recent reports by others that *PIK3CA* mutational status in breast cancer differs approximately 18% of the time between primary tumors and corresponding metastatic disease with changes in both directions (wild-type to mutant and mutant to wild-type) being observed (24, 25). Furthermore, our data suggest that this discordance is reflective of tumor evolution and not due to technical issues or platform selection as tissue DNA assessed for *PIK3CA* mutations by both standard sequencing and BEAMing showed 100% concordance. It has been assumed that clonal evolution in progressing advanced disease results from a gain of mutations for instance as a result of treatment selection, though spontaneous loss and gains of mutations have also been reported (26, 27). Tumor heterogeneity and sampling issues may also account for some of the previously reported findings, as a single biopsy using only micron thin sections for DNA analysis may not be representative of the whole tumor within a particular site or across metastatic sites (28–30). There is also evidence to suggest that multiple genetically diverse clonal subpopulations exist within primary breast cancers, in contrast to previously accepted models of tumor progression and metastatic dissemination punctuated by clonal expansions (31). It is therefore tempting to speculate that the finding of *PIK3CA* mutations in an original primary archival tumor that then "converts" to wild-type *PIK3CA* status at the time of recurrence or metastasis might represent the emergence of a new population of drug resistant clones and/or clones with increased metastatic potential.

A strength of the BEAMing technology is the ability to detect rare mutant molecules present in any source of DNA. A notable limitation is that BEAMing can detect only known mutations that have been optimized for the assay before sample analysis, that is, BEAMing currently cannot be used for mutation discovery. BEAMing is therefore ideally suited for genes with common recurrent "driver" mutations such as the *PIK3CA* mutations described here, as well as *BRAF* V600E and *KRAS* codon 12 and 13 mutations, with potential use in current clinical practice for targeted cancer therapies where these mutations have positive and negative predictive value (11–13). Other available assays can screen for higher numbers of somatic mutations such as the "SNaPshot" platform, which offers the possibility of screening more than 100 mutations. However, the limits of sensitivity of this technique for *PIK3CA* mutation detection is reported to be approximately 5% (5 mutant *PIK3CA* molecules per 100; ref. 32) compared with BEAMing which is capable of detecting at least one mutant molecule in 10,000 (15). Similarly, Board and colleagues have also described the detection of *PIK3CA* mutations in circulating free DNA using the Amplification Refractory Mutation System (ARMS; ref. 33). While their study showed in patients with metastatic breast cancer a high sensitivity and specificity for detecting *PIK3CA* mutations, no mutations were detected in *PIK3CA*-positive patients with operable disease. This may be due to the limits of detection of this technique, which have been reported to be 0.1% to 1% (34). In contrast, BEAMing is at least an order of magnitude more sensitive (0.01%) and this sensitivity can be improved with higher fidelity DNA polymerases (15, 18). In addition, though not a focus of the current study, BEAMing is quantitative unlike most current technologies. This aspect of BEAMing may allow for its use as a surrogate marker of disease burden. As example, we have recently found that

Published OnlineFirst on March 15, 2012; DOI:10.1158/1078-0432.CCR-11-2696

PIK3CA mutations can be detected in early, patients with nonmetastatic breast cancer before surgery (unpublished observations). Following surgery, we would expect that ctDNA levels would dramatically decrease unless there is the presence of occult disease. These studies are currently ongoing and the basis for future analysis on the use of BEAMing for early-stage disease.

To our knowledge, this is the first prospective study evaluating the feasibility of BEAMing to identify oncogenic *PIK3CA* mutations from plasma-derived ctDNA. Our results suggest that the characterization of *PIK3CA* mutational status by testing a blood sample using BEAMing in patients with metastatic breast cancer is highly feasible. We have shown that BEAMing of plasma ctDNA correlates 100% with mutational status of a metastatic tumor specimen, when both samples are collected synchronously. If indeed PI3K inhibitors are shown to offer the greatest benefit in patients whose tumors harbor a *PIK3CA* mutation, our results and those of others suggest that patients should optimally be selected for these trials based on *PIK3CA* mutational status at the time of enrollment, rather than on mutational status of archival tissue. BEAMing offers a reliable, noninvasive blood test to assess *PIK3CA* mutational status that could be theoretically conducted in lieu of a biopsy making it highly attractive to patients and health care providers.

Disclosure of Potential Conflicts of Interest

F. Diehl and P. Angenendt are employees and stakeholders of Inostics GmBH who conducted BEAMing analyses. K.E. Bachman and J. Greshock are employees of GlaxoSmithKline. L.A. Emens has a commercial research grant for Genentech Incorporation and is a consultant/advisory board member for Genentech Incorporation, Bristol Myers Squibb, and Roche. B.H. Park is a consultant/advisory board member for GlaxoSmithKline and Horizon Discovery, Ltd. No potential conflicts of interest were disclosed by the other authors.

Grant Support

This work was supported in part by the Department of the Defense Breast Cancer Research Program W81XWH-10-1-0244; Susan G. Komen for the Cure PDF0707944, SAC110053; The Avon Foundation; NIH/National Cancer Institute CA009071, CA088843 (Breast SPORE), CA121937, CA109274, and the Breast Cancer Research Foundation.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Received October 19, 2011; revised February 3, 2012; accepted February 28, 2012; published OnlineFirst March 15, 2012.

References

1. Kalinsky K, Jacks LM, Heguy A, Patil S, Drobnjak M, Bhanot UK, et al. PIK3CA mutation associates with improved outcome in breast cancer. Clin Cancer Res 2009;15:5049–59.
2. Janku F, Tsimberidou AM, Garrido-Laguna I, Wang X, Luthra R, Hong DS, et al. PIK3CA mutations in patients with advanced cancers treated with PI3K/AKT/mTOR axis inhibitors. Mol Cancer Ther 2011;10:558–65.
3. Baselga J, De Jonge MJ, Rodon J, de Jonge M, Verweij J, Birle D, et al. A first-in-human phase I study of BKM120, an oral pan-class I PI3K inhibitor, in patients (pts) with advanced solid tumors. J Clin Oncol 28:15s, 2010 (suppl; abstr 3003).
4. Tanaka H, Yoshida M, Tanimura H, Fujii T, Sakata K, Tachibana Y, et al. The selective class I PI3K inhibitor CH5132799 targets human cancers harboring oncogenic PIK3CA mutations. Clin Cancer Res 2011;17:3272–81.
5. O'Brien C, Wallin JJ, Sampath D, GuhaThakurta D, Savage H, Punnoose EA, et al. Predictive biomarkers of sensitivity to the phosphatidylinositol 3′ kinase inhibitor GDC-0941 in breast cancer preclinical models. Clin Cancer Res 2010;16:3670–83.
6. Bachman KE, Argani P, Samuels Y, Silliman N, Ptak J, Szabo S, et al. The PIK3CA gene is mutated with high frequency in human breast cancers. Cancer Biol Ther 2004;3:772–5.
7. Saal LH, Holm K, Maurer M, Memeo L, Su T, Wang X, et al. PIK3CA mutations correlate with hormone receptors, node metastasis, and ERBB2, and are mutually exclusive with PTEN loss in human breast carcinoma. Cancer Res 2005;65:2554–9.
8. Karakas B, Bachman KE, Park BH. Mutation of the PIK3CA oncogene in human cancers. Br J Cancer 2006;94:455–9.
9. Lynch TJ, Bell DW, Sordella R, Gurubhagavatula S, Okimoto RA, Brannigan BW, et al. Activating mutations in the epidermal growth factor receptor underlying responsiveness of non-small-cell lung cancer to gefitinib. N Engl J Med 2004;350:2129–39.
10. Sequist LV, Bell DW, Lynch TJ, Haber DA. Molecular predictors of response to epidermal growth factor receptor antagonists in non-small-cell lung cancer. J Clin Oncol 2007;25:587–95.
11. Amado RG, Wolf M, Peeters M, Van Cutsem E, Siena S, Freeman DJ, et al. Wild-type KRAS is required for panitumumab efficacy in patients with metastatic colorectal cancer. J Clin Oncol 2008;26:1626–34.
12. Karapetis CS, Khambata-Ford S, Jonker DJ, O'Callaghan CJ, Tu D, Tebbutt NC, et al. K-ras mutations and benefit from cetuximab in advanced colorectal cancer. N Engl J Med 2008;359:1757–65.
13. Flaherty KT, Puzanov I, Kim KB, Ribas A, McArthur GA, Sosman JA, et al. Inhibition of mutated, activated BRAF in metastatic melanoma. N Engl J Med 2010;363:809–19.
14. Aung KL, Board RE, Ellison G, Donald E, Ward T, Clack G, et al. Current status and future potential of somatic mutation testing from circulating free DNA in patients with solid tumours. Hugo J 2011;4:11–21.
15. Diehl F, Li M, Dressman D, He Y, Shen D, Szabo S, et al. Detection and quantification of mutations in the plasma of patients with colorectal tumors. Proc Natl Acad Sci U S A 2005;102:16368–73.
16. Dressman D, Yan H, Traverso G, Kinzler KW, Vogelstein B. Transforming single DNA molecules into fluorescent magnetic particles for detection and enumeration of genetic variations. Proc Natl Acad Sci U S A 2003;100:8817–22.
17. Diehl F, Li M, He Y, Kinzler KW, Vogelstein B, Dressman D. BEAMing: single-molecule PCR on microparticles in water-in-oil emulsions. Nat Methods 2006;3:551–9.
18. Diehl F, Schmidt K, Choti MA, Romans K, Goodman S, Li M, et al. Circulating mutant DNA to assess tumor dynamics. Nat Med 2008;14:985–90.
19. Distribution of Somatic Mutations in PIK3CA. Catalogue Of Somatic Mutations in Cancer (COSMIC). Cambridge, UK: Wellcome Trust Genome Campus; 2011 [cited 2011]. Available from: http://www.sanger.ac.uk/genetics/CGP/cosmic/
20. Maruyama N, Miyoshi Y, Taguchi T, Tamaki Y, Monden M, Noguchi S. Clinicopathologic analysis of breast cancers with PIK3CA mutations in Japanese women. Clin Cancer Res 2007;13:408–14.
21. Campbell IG, Russell SE, Choong DY, Montgomery KG, Ciavarella ML, Hooi CS, et al. Mutation of the PIK3CA gene in ovarian and breast cancer. Cancer Res 2004;64:7678–81.
22. Stemke-Hale K, Gonzalez-Angulo AM, Lluch A, Neve RM, Kuo WL, Davies M, et al. An integrative genomic and proteomic analysis of PIK3CA, PTEN, and AKT mutations in breast cancer. Cancer Res 2008;68:6084–91.
23. Li M, Diehl F, Dressman D, Vogelstein B, Kinzler KW. BEAMing up for detection and quantification of rare sequence variants. Nat Methods 2006;3:95–7.

Published OnlineFirst on March 15, 2012; DOI:10.1158/1078-0432.CCR-11-2696

24. Dupont Jensen J, Laenkholm AV, Knoop A, Ewertz M, Bandaru R, Liu W, et al. PIK3CA mutations may be discordant between primary and corresponding metastatic disease in breast cancer. Clin Cancer Res 2011;17:667–77.
25. Gonzalez-Angulo AM, Ferrer-Lozano J, Stemke-Hale KA, Sahin A, Liu S, Barrera JA, et al. PI3K pathway mutations and PTEN levels in primary and metastatic breast cancer. Mol Cancer Ther 2011;10: 1093–101.
26. Bouchahda M, Karaboue A, Saffroy R, Innominato P, Gorden L, Guettier C, et al. Acquired KRAS mutations during progression of colorectal cancer metastases: possible implications for therapy and prognosis. Cancer Chemother Pharmacol 2010;66: 605–9.
27. Badalian G, Barbai T, Raso E, Derecskei K, Szendroi M, Timar J. Phenotype of bone metastases of non-small cell lung cancer: epidermal growth factor receptor expression and K-RAS mutational status. Pathol Oncol Res 2007;13:99–104.
28. Park SY, Gonen M, Kim HJ, Michor F, Polyak K. Cellular and genetic diversity in the progression of *in situ* human breast carcinomas to an invasive phenotype. J Clin Invest 2010;120:636–44.
29. Torres L, Ribeiro FR, Pandis N, Andersen JA, Heim S, Teixeira MR. Intratumor genomic heterogeneity in breast cancer with clonal divergence between primary carcinomas and lymph node metastases. Breast Cancer Res Treat 2007;102:143–55.
30. Yachida S, Jones S, Bozic I, Antal T, Leary R, Fu B, et al. Distant metastasis occurs late during the genetic evolution of pancreatic cancer. Nature 2010;467:1114–7.
31. Navin N, Kendall J, Troge J, Andrews P, Rodgers L, McIndoo J, et al. Tumour evolution inferred by single-cell sequencing. Nature 2011;472: 90–4.
32. Hurst CD, Zuiverloon TC, Hafner C, Zwarthoff EC, Knowles MA. A SNaPshot assay for the rapid and simple detection of four common hotspot codon mutations in the PIK3CA gene. BMC Res Notes 2009;2:66.
33. Board RE, Wardley AM, Dixon JM, Armstrong AC, Howell S, Renshaw L, et al. Detection of PIK3CA mutations in circulating free DNA in patients with breast cancer. Breast Cancer Res Treat 2010;120:461–7.
34. Board RE, Thelwell NJ, Ravetto PF, Little S, Ranson M, Dive C, et al. Multiplexed assays for detection of mutations in PIK3CA. Clin Chem 2008;54:757–60.